



FIG. 7

EP 0 506 124 A1

Field of the invention

The invention relates to a composition for enhancing delivery of a therapeutic agent to a solid tissue site, comprising: two single chain V_H - V_L bifunctional binding molecules which are joined together, having one specificity for a solid tissue antigen and the other for the therapeutic agent; a remover substance which binds circulating binding molecule; a therapeutic agent.

When the composition of the invention including two-domain binding molecules is used to recruit a therapeutic agent to a solid tissue site, the bifunctional binding molecules are administered first, and once the binding molecules reach maximum concentration in the extravascular space, a remover substance is administered to aid in clearing the binding molecules in the blood circulation and extravascular space, and thereafter, the therapeutic agent is administered.

Background of the invention

Much research and experimentation has been done on how to deliver therapeutic and imaging agents to solid tissue sites *in vivo*. Such site-specific delivery has often been attempted with monoclonal antibodies ("mAbs") conjugated with the therapeutic or imaging agents. These immunoconjugates are often called "magic bullets", because of their ability to specifically target diseased or tumorous sites *in vivo*.

Immunotoxins, which are immunoconjugates in which mAbs are conjugated with toxic substances, such as plant or bacterial-derived toxins including *Pseudomonas* exotoxin, ribosomal-inactivating proteins, ricin, gelonin, and pokeweed antiviral peptide, have also been extensively studied. Additionally, mAbs conjugated with metal-chelating agents, where the metal-chelating agents can carry radioactive isotopes, have been used for both treating and imaging tumors.

Immunoconjugates, and particularly immunotoxins, have been actively investigated for treatment of tumors both in solid tissue and in other areas. Clinical trials of immunotoxins for removing tumors or decreasing tumor loads have been conducted. Such tests have often been with immunotoxins where the mAb is conjugated with the A chain of ricin or a radioactive isotope. Immunotoxins have also been studied in animal models for eliminating malignant cells in tumors transplanted into the animals.

These studies indicate that immunotoxins are more effective in treating leukemia or lymphoma than solid tumors. One plausible explanation for this difference in efficacy is that malignant cells in blood or lymphoid tissues are more accessible

than those in solid tumors. Thus, many malignant cells in a solid tumor come in contact only with insufficient amounts of toxin to kill them. In addition, even where the toxin is in contact with the target cells, only a very small fraction will actually enter the cell and thus, not all cells in a solid tumor will be killed.

It is possible, of course, to increase the total amount of immunotoxin administered, in order to increase that which is in the vicinity of malignant cells and available to kill the cells. However, because of the conjugation with the antibody molecules, much of the immunotoxin is also absorbed and taken up by the reticuloendothelial cells of the body. The toxin will damage or destroy these cells. Specifically, a large proportion of immunotoxin ends up in the phagocytic cells in the liver, where, because of its toxicity, it can damage the liver and its function. Thus, the total amount of toxin which can be administered is severely limited.

An illustration of the problems encountered with immunotoxins is seen in a typical clinical trial. See Parker, S.A. et al. "Therapeutic Monoclonal Antibodies" Ed. by Borrebaeck, C.A. and Larrick, J.W. pp. 127-141 (Stockton Press, New York 1990). Patients with B cell lymphoma were treated with anti-idiotypic antibodies coupled with the radioactive isotope $^{90}\text{Yttrium}$. This therapy proved so toxic that the immunoconjugate had to be administered with excess cold, unlabeled anti-idiotypic antibodies. However, the excess cold anti-idiotypes competed with the labeled immunoconjugates for binding to the tumor associated antigen, and thereby inhibited the binding of the immunoconjugates to the tumor cell targets.

Similar drawbacks result where an immunoconjugate which includes a mAb and a radioactive isotope is used for tumor imaging. The immunoconjugate tends to be bound and taken up in phagocytic cells in the liver, spleen, and blood circulation, because the antibody portion of the immunoconjugate is absorbed by these cells. This increases the background "noise" and interferes with tumor imaging, and it can also cause toxic levels of radioactivity in all of these organs.

Several groups have tried to solve the major problem which results when using mAbs coupled with imaging agents, i.e., the imaging agent is absorbed *in vivo* and cleared together with the antibody. One group suggested that instead of coupling the mAbs and the imaging agents, a bispecific antibody, which is not coupled to an imaging agent, should be administered first. The bispecific antibody has one specificity against the tumor being targeted and the other against a chelate conjugated to a peptide. The bispecific antibody distributes between the tumor and the circulation, and at a point when there is a high

tumor-to-background ratio, a labeled chelate is administered. The chelate which is not absorbed by the antibody is rapidly excreted by the kidneys, due to its relatively small size. This results in low background noise. See *Monoclonal Antibodies in Immunoscintigraphy* Ed. by Chatal, J.-F., pp. 70-71 (CRC Press, Boca Raton Fla. 1989).

Another group discussed administering an antibody which slowly diffuses to the target tumor, and then clearing the excess circulating antibody. The clearance is done with an antigen covalently bound to a slowly diffusable serum protein (human transferrin). Thereafter, the imaging tracer is administered as an epitopically derivatized bifunctional chelate which is small and rapidly diffusable, and quickly cleared. Again, this is designed to help reduce background radiation and improve imaging. See Goodwin, D.A. et al., *J. Nuc. Med.* 29:228-34 (1988). A related paper suggested using bifunctional antibodies such as two Fab' fragments coupled at the SH groups, where one specificity is for the chelate and the other is for the tumor site antigen. See Goodwin, D.A., *J. Nuc. Med.* 28:1359-62(1987).

Another related paper suggested injecting antibody and labeled protein (transferrin) followed by injection of anti-human IgG antibody and anti-transferrin antibody. The second antibody injection helps to clear excess labeled transferrin and reduce the background noise. See Goodwin, D.A. et al., *J. Nuc. Med.* 9:209-215(1984).

None of these articles discuss how to clear both the blood vessels and the extravascular space of binding molecules prior to administering the imaging agents, while retaining, attached to the target tissue, as much as possible of the binding molecules. When administering toxins or therapeutic agents, it is even more important to clear binding molecules from the extravascular space (as well as from the blood vessels) so that excess toxin is not bound by the binding molecules in the extravascular space and does not cause damage. Thus, if bispecific binding molecules with one specificity for the target site and one for the toxin/therapeutic agent are administered initially, those molecules which bind to the target site must be retained as much as possible, and those molecules which are unbound and in the circulation or extravascular space should be removed. It is also important that the removal should be accomplished quickly enough so that the binding molecule is not released from the target site before the toxin/therapeutic agent is administered.

A number of factors must be considered in designing an effective method of treating solid tumors using tissue-specific recruiting by a binding molecule of a therapeutic agent. These factors include:

- 1) the pharmacokinetic properties of the binding molecules, therapeutic agents, and other substances used in the method;
- 2) the clearance routes (reticuloendothelial system versus kidney) of the binding molecules, therapeutic agents and other substances;
- 3) the diffusion rates of the binding molecules and therapeutic agents in and out from the capillaries;
- 4) the binding molecules must not be endocytosed by the cells;
- 5) the on/off times of the binding molecules on the target cells;
- 6) the affinity of the binding molecules for the therapeutic agents, and the efficiency with which they can recruit the therapeutic agents to the target site;
- 7) the therapeutic cytotoxins such as ricin A chain, pokeweed antiviral peptide, must enter the target cells to render effects, whereas some other therapeutic substances (and imaging agents) need not enter the target cells to be effective;
- 8) the immunogenicity and antigenicity of the binding molecules and the therapeutic agents.

These factors make designing an effective method very complex.

Summary of the invention

The invention provides a composition for enhancing delivery of a therapeutic agent to a solid tissue site, comprising: two single chain V_H - V_L bifunctional binding molecules which are joined together, having one specificity for a solid tissue antigen and the other for the therapeutic agent; a remover substance which binds circulating binding molecule; a therapeutic agent.

The bifunctional two-domain binding molecules of the invention are effective for recruiting a therapeutic agent to a solid tissue target site, as the binding molecules have one specificity for the target site and the other specificity for the therapeutic agent. The therapeutic agent is administered separately, after administering the binding molecules and after administering a remover substance.

The remover is preferably a liposome which is conjugated with antibodies against the binding molecules. The remover cannot diffuse into the extravascular space and is rapidly removed by the phagocytic cells in the liver, spleen and blood circulation. It binds to binding molecules which are in the circulation, which thereby facilitates the clearing of the binding molecules from the circulation. After clearance, there is a concentration difference in binding molecules across the blood vessel wall, and binding molecules in the extravascular space diffuse into the blood vessels.

The remover should be administered as soon as possible after the binding molecules reach a maximum concentration in the vascular space. When administered at such time, substantial amounts of binding molecule have not yet been released from the target tissue site.

The remover is preferably administered at least twice, and the subsequent administrations of the remover are at a time after the binding molecules in the circulation and extravascular space have reached equilibrium. The administration of remover effectively clears the majority of the binding molecule in both the extravascular space and in the circulation, and will therefore further increase the ratio of binding molecules in the target site over binding molecules in the circulation and extravascular space.

The therapeutic agent should be administered after the last administration of the remover, and after the remover has had enough time to clear from the circulation. But the therapeutic agent should be administered before substantial amounts of binding molecules are released from the target site, so that as much of the therapeutic agent as possible will be bound at the target tissue site by the binding molecules.

The bifunctional two-domain binding molecules are preferably two V_H - V_L single-chain binding molecules which are joined together. They can be joined with a linking peptide, as described in International Application No. WO88/08344. See, e.g., Fig. 2D. It is possible to design the remover to include anti-idiotypic antibodies which recognize one or more of the binding sites of the binding molecules. In a preferred embodiment, however, an antibody conjugated to the remover recognizes an antigenic structure associated with the joining region between the two V_H - V_L single-chain binding molecules, or the antibody recognizes the linking peptide itself. Alternatively, the peptide joining the two V_H - V_L single-chain binding molecules may be glycosylated, and may have a non-glycosylated peptide or hapten attached thereto. The non-glycosylated peptide is specifically recognized by the antibodies associated with the remover.

It is also preferred, for those therapeutic agents which must enter the cell in order to be effective, that they be linked to a peptide blocker which prevents the therapeutic agent from entering a cell. The blocker is preferably bound by the binding molecules.

The preferred means for linking such therapeutic agents with a blocker is with a hydrophobic, lipophilic peptide linker, such as that described in International Application No. PCT/US88/03632. There should also be a cleavage site between the blocker and the therapeutic agent, so that the blocker can be cleaved and allow the therapeutic agent

to enter the cell.

The immunoconjugate of the therapeutic agent, the linker and the blocker should be small to minimize the phagocytosis by reticuloendothelial system ("RES") cells. Preferably, during the time the blocker is bound at the target site by the binding molecules, the blocker will be cleaved and released. The hydrophobic, lipophilic linker will tend to blend with the cell membrane, and thereby enhance the entry of the therapeutic agent into the cell.

The invention will now be described in further detail with reference to the drawings.

Brief Description of the Drawings

Fig. 1, which plots binding molecule concentration in the circulation against time, shows the kinetics of binding molecule concentration in the circulation and the extravascular space, after intravenous administration.

Fig. 2 shows the kinetics of binding molecule concentration at the tumor site and in the circulation, after intravenous administration.

Fig. 3 is a schematic representation of a blood vessel surrounded by an extravascular space which is in turn contacted by solid tissue. The solid tissue has a tumor thereon, as indicated. The liver is shown schematically as fluidly linked with the blood. Binding molecules (the solid dots inside the blood vessel) have been administered.

Fig. 4 is the same schematic representation as in Fig. 3, but at a later time when the binding molecules have reached a maximum concentration in the extravascular space.

Fig. 5 is the same schematic representation as in Fig. 4, but after administration of remover substance.

Fig. 6 is the same schematic representation as in Fig. 5, but at a later time after much of the binding molecules have cleared from the circulation.

Fig. 7 is a schematic representation of a bifunctional two-domain binding molecule, suitable for use with the invention.

Fig. 8 shows the bifunctional two-domain binding molecule of Fig. 7 with an antigenic peptide attached to the glycosylated chain on the linker.

Fig. 9 is a schematic representation of one preferred form of the therapeutic agent, having a therapeutic substance attached with a membrane translocation agent to a blocker.

Detailed Description of the Invention

In the preferred mode of the invention, therefore, three different substances are introduced in three phases. First, bifunctional two-domain binding

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molecules, with one specificity for antigens at the target site and one specificity for an antigenic site on the therapeutic agent, are administered and allowed time to bind to the target site and to reach a maximum concentration in the extravascular space. The concentrations of the binding molecules in the extravascular space can be monitored by assaying the binding molecules in fluid samples drawn from the peritoneal cavity. Once the binding molecules reach maximum concentration in the extravascular space, a remover substance, preferably a liposome conjugated with antibodies, is administered to remove excess binding molecules from the circulation. This creates a difference in binding molecule concentration across the blood vessel wall. The binding molecules in the extravascular space will tend to diffuse into the blood vessels, where they will also be bound by the remover substance.

The remover is preferably re-introduced several times, each introduction being immediately after the binding molecules reach equilibrium across the blood vessel wall. Each introduction of remover depletes the binding molecules in the blood vessel, because once the binding molecules are bound by the remover they are more easily and rapidly cleared by the RES. The lowered concentration of binding molecules in the blood vessels causes diffusion of the binding molecules from the extravascular space into the blood vessels, resulting in a continuous decrease in the concentration of binding molecules in the blood vessel and the extravascular space. Because the binding molecules are not conjugated with the therapeutic cytotoxin the uptake and removal of the binding molecules by RES does not poison the cells.

A therapeutic agent is then administered, preferably immediately after the last administration of remover and before any significant amount of the binding molecules are released from the target site. The therapeutic agent is bound at the target site by the binding molecules, which have one specificity for an antigenic site associated with the therapeutic agent.

It is preferred if the entire procedure is completed in eight hours or less. This is a short enough time to prevent substantial amounts of binding molecules from releasing from the target site before the therapeutic agent is administered.

In conventional immunotoxin therapy, the immunotoxin conjugate made up of the antibody and the toxin must be endocytosed by the target cell to kill the cell. One important difference of the present invention is that the bifunctional two-domain binding molecules of the present invention must not be endocytosed by the target cells. If the binding molecules of the invention are endocytosed, they will not be available to bind the therapeutic agent

when it is administered.

The bifunctional two-domain binding molecules avoid endocytosis by the target cells because they have only one valency for the tumor-associated antigen, and thus should not induce cross-linking of the antigen. Cross-linking generally will induce endocytosis.

The advantages of the invention include that fact that it maximizes the amount of binding molecule which is at the target site at the time the therapeutic agent is administered, and thereby maximizes the amount of therapeutic agent which reaches the target site. This is achieved through use of small bifunctional two-domain binding molecules and proper timing of the administration of the remover and the therapeutic agent.

The small bifunctional two-domain binding molecules diffuse relatively quickly through the capillary walls, so that administering the remover effectively clears both the circulation and the extravascular space of excess binding molecules before significant amounts of the binding molecules can be released from the target site. This results in less toxicity to the reticuloendothelial cells and other cells in the circulation, and less toxicity to the phagocytic cells in the liver, because the therapeutic agent is bound at the target site and does not circulate or get absorbed in undesired locations.

The preferred binding molecules have the following properties:

- 1) They have a very high affinity for the target site surface antigen, with a K_a above 1×10^8 mole⁻¹;
- 2) They have a very high affinity for the therapeutic agent, with a K_a above 1×10^8 mole⁻¹;
- 3) They have relatively fast kinetic properties, i.e., they reach equilibrium between the blood vessel and the extravascular space relatively quickly (time " $t_{1/2}$ " in Figs. 1 and 2 is relatively short);
- 4) They are not appreciably endocytosed by the target cells, after they bind to the target cells' surface antigen.

The preferred bifunctional two-domain binding molecules for use with the invention are two V_H-V_L single chain binding molecules joined together, as described in International Application No. WO88/08344, and as schematically shown in Fig. 7. For binding molecule 30 of Fig. 7, a linker 32 joins the one V_H-V_L single-chain binding domain 34 to the other V_H-V_L single-chain binding domain 36.

Single-chain V_H-V_L binding molecules (as distinct from the two single chain binding molecules of the invention) consist of the Fv portion of an antibody light and heavy chain linked together, typically with a short peptide chain. See U.S. Patent No. 4,948,778. In the invention, with two single-chain V_H-V_L binding molecules joined together, one

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in length. Peptide 42 provides an antigenic site for the antibody associated with the remover.

The preferred remover substance has the following properties:

- 1) It can specifically bind to the binding molecules;
- 2) It remains in the circulation and does not diffuse through the holes in the capillary wall and into the extravascular space or into solid tissues, except that it does diffuse into the spleen, liver and lymphoid tissues;
- 3) It is rapidly cleared by the reticuloendothelial system.

The preferred remover is a liposome which is conjugated with antibodies specific for an antigenic site associated with the peptide which joins the two bifunctional binding molecules together. However, the antibodies can also be specific for any portion of the linked V_H - V_L binding molecules, or they can be anti-idiotypes to the V_H - V_L binding molecules. The antibodies can also be conjugated to a polymeric substance, such as dextran or polyethylene glycol.

Referring to Figs. 1 and 2, the pharmacokinetics of a binding molecule which has been administered is illustrated. Curve A in Fig. 1 represents the concentration of binding molecule in the blood circulation, and curve B represents the concentration in the extravascular space. Curve A shows a rapid decline after injection to time t''_{max} , representing the time during which binding molecules diffuse into the extravascular space and are bound at the target site. After time t''_{max} , curve A declines more slowly, representing the clearance of the binding molecules by the reticuloendothelial system, the kidneys and other cells of the body.

Referring to curve B in Fig. 1, it can be seen that the concentration of binding molecule in the extravascular space increases after injection to t_{max} , when equilibrium between the blood circulation and the extravascular space is reached.

Fig. 2 represents the kinetics of distribution of the binding molecules between the tumor and the blood. Curve A represents the amount of binding molecule associated with the target antigen. It increases rapidly from injection to time t''_{max} , and then declines slowly thereafter, the latter phase representing the time during which binding molecules are releasing from the target site. Curve B represents the tumor/blood concentration ratio. It can be seen that this ratio increases slightly more rapidly from injection to time t''_{max} than the increase after time t''_{max} .

When the binding molecules administered are smaller, the pharmacodynamic consequences are as follows:

- 1) the time to $t_{max}^{(1)}$ is shorter;
- 2) the concentration difference between the

- blood and the extravascular space is smaller;
- 3) the concentration of the binding molecules in the extravascular space can reach higher levels;
- 4) more of the binding molecules can be bound at the target site;
- 5) the binding molecules diffuse more quickly from the extravascular space to the blood circulation.

Because all these consequences are advantageous, the preferred binding molecules are two single-chain V_H - V_L binding molecules joined together. Such joined single chain binding molecules are smaller than many antibody fragments, such as $F(ab')_2$ fragments, and smaller than whole antibodies.

The clearance of binding molecules at different points in time in relation to the administration of the remover is illustrated in Figs. 3-7. Fig. 3 schematically shows a blood vessel 10 surrounded by extravascular space 12 which is in turn contacted by solid tissue 14. The solid tissue 14 has a tumor 16 thereon, which is a target site for the binding molecules 20. The liver 18 is shown fluidly linked with the blood vessel 10. Binding molecules 20 have recently been administered and are still all within the blood vessel 10.

Fig. 4 shows the same view as Fig. 3, shortly thereafter. Fig. 4, represents time t^{**}_{max} of Figs. 1 and 2, at which the binding molecules 20 reach a maximum concentration in the extravascular space 12 as the equilibrium between the extravascular space 12 and the inside of the blood vessel 10 is reached. The binding molecules 20 also bind to the target site at near maximum levels. Some of the binding molecules 20 have been absorbed and taken up by the liver 18.

Fig. 5 is at a later time, after a remover has been administered. As a result of the remover substance's action, the concentration of the binding molecules 20 in the blood vessel 10 is greatly reduced. Because the rate of diffusion of the binding molecules 20 across the capillary wall is slower than the rate of clearance of the remover by the RES, the concentration of binding molecules 20 is higher in the extravascular space 12 than in the blood vessel 10. The amount of binding molecules 20 bound by tumor 16 remains near maximum levels. More of the binding molecules 20 have been taken up by the liver 18.

Fig. 6 is at a still later time, after the binding molecule removal phase is substantially complete. Little of the binding molecules 20 remain in the blood vessel 10 or in the extravascular space 12. The amount of binding molecules 20 bound by the tumor 16 remains at about the same level as in Fig. 5, because the release time of the binding molecules is much longer than the time needed for the binding molecules to diffuse into the blood vessel

10 and be removed. The liver 18 has taken up more of the binding molecules 20 and has also digested some of them. The conditions are now optimal for administering the therapeutic agent.

Because the remover is intended to clear the free binding molecules in both the blood circulation and also in the extravascular space, it should be administered repeatedly shortly after the binding molecules reach t^{**}_{max} . It is more preferred if the remover is administered over a total length of time that is about 4-5 times t^{**}_{max} .

The preferred total length of the removal phase of 4-5 times t^{**}_{max} allows nearly all of the binding molecules to diffuse back into the blood vessels from the extravascular space, and to then be bound in the blood vessels by the remover substance. But such a removal phase is not so long as to allow significant amounts of the binding molecules to release from the target site before the therapeutic agent is administered.

It is preferred if the remover substance is either repeatedly added or is continuously infused intravenously. Assuming that at t^{**}_{max} about one-half of the binding molecules are in the extravascular space (which would result at equilibrium), then after the remover has been administered four times (at $4 \times t^{**}_{max}$), the residual amount of binding molecules in the extravascular space will be about $1/2 \times (1/2)^4$, or about 3% of the total binding molecules originally administered.

From the available pharmacokinetic data about whole IgG, and about $F(ab')_2$ and Fab fragments, it is estimated that t^{**}_{max} for whole IgG, $F(ab')_2$ and Fab are about 50 hours, 20 hours, and 1 hour, respectively. See LoBuglio, A.F. et al. *Proc. Natl. Acad. Sci. U.S.A.* 86:4220-4224 (1989); Moblotfsky, P.J. et al. *Radiology* 149:549-555 (1983); Larson, S.M. et al. *Radiology* 155:487-492 (1985). The two joined single-chain V_H - V_L binding molecules preferred for use with the invention are about the same size and have a similar overall structure to Fab fragments. Thus, these binding molecules should have about the same diffusion rate and t^{**}_{max} as the Fab fragments. Based on a t^{**}_{max} of 1 hour with the preferred binding molecules, the total time to complete administration of the binding molecules, the remover substance and the therapeutic agent is about 6 hours.

Bifunctional IgG or $F(ab')_2$ are not preferred for use with the invention because their diffusion rates are slow and their t^{**}_{max} is long. By the time the free binding molecules in the circulation and in the extravascular space are cleared by the remover agent (a period of several times t^{**}_{max}), significant amounts of binding molecule will have released from the target site. Thus, not as much of the therapeutic agent will be bound at the target site as when using the preferred binding molecules.

The blocker is described in International Application PCT/US89/03532 as preferably being an antibody which targets the particular cells sought to be treated. In the present invention there is no need for using an antibody as the blocker, as it is the single chain V_H - V_L binding molecules, not the blocker, which are responsible for the tissue-spe-

Another advantage of the invention is that the effectiveness of the therapeutic agent depends only on its concentration at the target site. A conventional immunotoxin must be endocytosed for it to be effective in killing target cells. This means that the entire structure, including the antibody and the toxin, must be endocytosed. As noted above,

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endocytosis is more likely to occur if the antibody can cross-link a target site antigen. However, it is well-known that the density of tumor-associated antigens on the cell surface varies from cell to cell, due to the cell's cycle, antigenic drift, and other factors. Thus, a conventional immunotoxin's effectiveness is limited to the extent to which it is endocytosed.

In contrast, the therapeutic agents of the present invention do not need to be endocytosed to be effective. For those therapeutic agents noted above which must enter the cell to be effective, the membrane blending agent aids their entry into the cell. Cross-linking of a surface antigen is not necessary, and therefore, their effectiveness is not limited by the availability of the cell surface antigens on a particular cell in the tissue site, but only by the total amount of the surface antigen at the tissue site.

Specific examples of making the various components of the invention are described below.

(1) Preparation of Conjugates of a Therapeutic Agent with Membrane Blending Agents and Blockers

A preferred therapeutic agent to be used in the form of molecular conjugates of the present invention is ricin A chain, which possesses the ribosome-inactivating activity but not the cell-binding activity of whole ricin molecules. The preparation of three-component molecular conjugates is described in International Application PCT/US88/03532.

For making these conjugates, a preferred group of membrane blending agents are long chain fatty acids. For convenience, fatty acids of 14, 16 or 18 carbons in length, more preferably having at least one double-bond for addition/substitution reactions, may be used. These fatty acids, namely myristoleic acid, palmitoleic acid, and oleic acid, which all have double bond at C₅-C₆, may be purchased from Matreya, Inc., in Pleasant Gap, Pennsylvania. The unsaturated double bond may be subjected to addition/substitution reactions to incorporate one of the many heterobifunctional cross-linking agents (which are available from reagents firms, e.g. Pierce Chemical Co.) using techniques which are routine in organic chemistry.

One possible group of blocking agents are haptens, such as 2,4,6-trinitrobenzene and phenylarsonate. Monoclonal antibodies against these hapten blocking agents have already been produced.

A preferred group of blocking agents are short peptides of about 6 to 10 amino acids in length, which do not bear any autologous antigenic epitopes present in humans. The peptides should be

resistant to proteolytic digestion in serum and other body fluids. The amino acid sequence may be checked using one of the available programs (e.g., the Micro Genie™ program from Beckman Instruments) for homology with the peptide sequence of human proteins, which is available in recently updated database. The peptides derived from proteins of animal or insect or microorganism origin are possible choices. One specific example of a suitable peptide is an eight amino acid segment (Thr-Leu-Pro-Ile-Ala-His-Glu-Asp) from the CH₂ domain of rabbit IgG, residues #324-331. Five or six of these eight amino acids are different between this segment and the corresponding segment of human IgG₁, IgG₂, IgG₃, and IgG₄.

Depending on the cross-linking agent to be used, a cysteine residue can be added to the N or C-terminal end of the peptide to aid in linking.

(2) Preparation of Monoclonal Antibodies Against Haptens or Short Peptides

In the present invention, monoclonal antibodies specific for haptens or short peptides are of two types:

(1) Monoclonal antibodies specific for the blocking groups of the molecular conjugates of therapeutic agents. These monoclonal antibodies can be re-engineered to make one binding domain of the bifunctional single-chain binding molecules of the invention.

(2) Monoclonal antibodies specific for the antigenic epitopes on the linking peptide of the bifunctional two-domain binding molecules of the invention. These monoclonal antibodies are preferably incorporated into liposomes to make the remover substances of the invention, or they can be conjugated with other larger molecules to make a suitable remover substance.

Monoclonal antibodies specific for haptens or short peptides can be prepared according to routine, standard methods for making hybridomas and monoclonal antibodies, such as the methods described in Kennell, R.H. et al (Eds.), *Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analysis*, pp. 353-417 (Plenum Press, New York 1980) or Hudson, L. and Hay, F.C. (Eds.), *Practical Immunology 2nd ed.* (Blackwell Scientific Publications, Boston 1980).

According to the procedures described in these handbooks, the hapten or the peptide can be conjugated to a protein carrier such as keyhole limpet hemocyanin (KLH) at a ratio of multiple peptides (hapten groups) per KLH molecule. Mice are immunized with the conjugate in complete Freund's adjuvant, intraperitoneally, in the first immunization, and in incomplete Freund's adjuvant in 3-4 subsequent immunizations. The spleen cells from the

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tumor-associated surface antigens in human pancreatic and colorectal cancers (e.g., antibody 17-1A), ovarian cancer (e.g., antibody against CA125), liver cancer (e.g., anti-CEA), breast cancer (e.g., monoclonal antibody 72.3), melanoma (e.g., antibody 48.7 against HMWA antigen), as well as against many other tumors, have been developed. Many of these antibodies have been well known in the field of human tumor immunotherapy and have been studied in human clinical trials. Cell lines derived from the tumorous tissues have also been developed. In one very useful animal model using nude mice, the human tumor cell lines, when transplanted to the mice, develop tumors. Thus, a candidate therapeutic antibody or binding molecule can be used experimentally in the mouse model.

For constructing a two binding domain single chain binding molecule, one of binding domain can be derived from the Fv of one of these anti-tumor surface-antigen antibodies. The other domain can be derived from the Fv of an antibody specific to the blocking agent of the therapeutic agent described above. The cloning and the sequencing determination of the VH, VL of these antibodies can be performed by routine molecular biology techniques.

A preferred specific sequence of a linker between the two binding domains of the single chain binding molecules of the present invention should contain a nonautologous segment. An example is: Gly*Gly*Ser*Thr*Pro*Ser*Pro*Gly*ile*Gln*Val*Ser*Gly*Gly

The underlined portion of eight amino acids is a segment in the CH3 domain of rabbit μ chain (#382-388). Six of eight amino acid residues are different from the corresponding segment in human μ chain. The flanking Gly and Ser residues are for increasing the total length to 14. The sequence does not contain glycosylation site and the entire DNA encoding the two binding domains and this linker can thus be expressed in *E. coli*.

Another preferred linker is a glycosylated form. The peptide portion contains a glycosylation site. One example of this linker is:

48 Gly* Gly* Ser* Asn* Gly* Ser* Gly* Gly* Asn* Gly* Thr*
r* Gly* Ser* Gly

The sequence contains two potential N-glycosylation sites (underlined). The Gly and Ser residues enhance the flexibility and non-rigid conformation of the linker.

The DNA encoding the two binding domains and the linker should be expressed in mammalian cells, such as CHO cell line, which can add the carbohydrate moiety to the glycosylation sites. One preferred expression system to be used in a CHO cell line for the expression of immunoglobulin genes is the method described by Page, M.J. and Svdanham, M.A. *Bio/Technology* 9:84-88 (1991).

In the following section, the conjugation of a hapten or a peptide to a carbohydrate moiety is described. The eight amino acid peptide described in this section can be used for conjugation to the carbohydrate moiety.

(5) Conjugation of an Antigenic Peptide or Hapten to the Carbohydrate Site of Two Linked Single Chain V_H-V_L Binding Molecules

The preferred method of conjugating an antigenic peptide or hapten to the carbohydrate site of two linked single chain V_H-V_L binding molecules is adopted from the procedures described by O'Shannessy, D.J. et al., *Immunol. Lett.* 8:273-277 (1984) and Rodwell, J.D. et al., *Proc. Nat'l. Acad. Sci. U.S.A.* 83:2632-2636 (1986). The principle is to generate reactive aldehydes on the sugar moieties by sodium periodate treatment, and to link a reactive hydrazide group of the bifunctional linking group to the hapten or peptide to be conjugated and then to couple the two reactants.

This procedure has been applied successfully to conjugate antigenic peptides and haptens to a number of different IgG and IgM monoclonal antibodies. For example, these techniques have been used by Cytogen Corp. to make immunoconjugates for imaging tumors *in vivo*, which are now pending FDA approval.

The terms, expressions and examples herein are exemplary only and not limiting, and those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. All such equivalents are encompassed by the following claims.

Claims

1. A composition for enhancing delivery of a therapeutic agent to a solid tissue site, comprising:
two single chain V_H-V_L bifunctional binding molecules which are joined together, having one specificity for a solid tissue antigen and the other for the therapeutic agent;
a remover substance which binds circulating binding molecules;
a therapeutic agent.
2. The composition of claim 1 wherein the remover is a liposome conjugated with antibodies specific for the binding molecules.
3. The composition of claim 1 or 2 wherein the therapeutic agent is selected from the group consisting of cytotoxic or cytolytic substances, including ricin A chain, modified *Pseudomonas*

exotoxin A (that lacks cell binding domain and possesses translocation and ADP ribosylation activity), gelonin, abrin, diphtheria toxin, pokeweed anti-viral peptide, trichothecenes, cytokines, including tumor necrosis factor ("TNF") and interleukin 1; anti-sense RNAs that inhibit the expression of tumorigenic proteins.

4. The composition of claim 1 or 2 where the therapeutic agent is ricin A chain or modified *pseudomonas* exotoxin A, conjugated with a membrane blending agent and a blocker.
5. Two single-chain binding molecules linked together by a small non-autologous hydrophilic peptide.
6. The linked binding molecules of claim 5 wherein the linker has a glycosylation sequence.
7. The linked binding molecules of claim 5 or 6 wherein the linker is glycosylated.
8. The linked binding molecules of any one of claims 5 to 7 wherein the linker is preferably of about 10 to about 15 amino acid residues in length.
9. The linked binding molecules of any one of claims 5 to 8 including the linker's carbohydrate moiety is linked to a non-glycosylated peptide which is non-autologous and antigenic.
10. A liposome having an antibody attached to it wherein the antibody is specific for an antigenic site associated with a linker between two single chain V_H-V_L binding molecules which are joined together.
11. The liposome of claim 10 wherein the antigenic site is on the peptide linker.
12. The liposome of claim 10 or 11 wherein the antigenic site is on a peptide chain attached to a carbohydrate moiety of the linker.
13. Two single chain V_H-V_L bifunctional binding molecules which are joined together, wherein one valency is for a tumor-associated antigen and the other is for a blocker which is linked via a membrane translocation agent to a therapeutic agent.

Fig 1

Distribution of binding molecules in circulation and in extravascular space

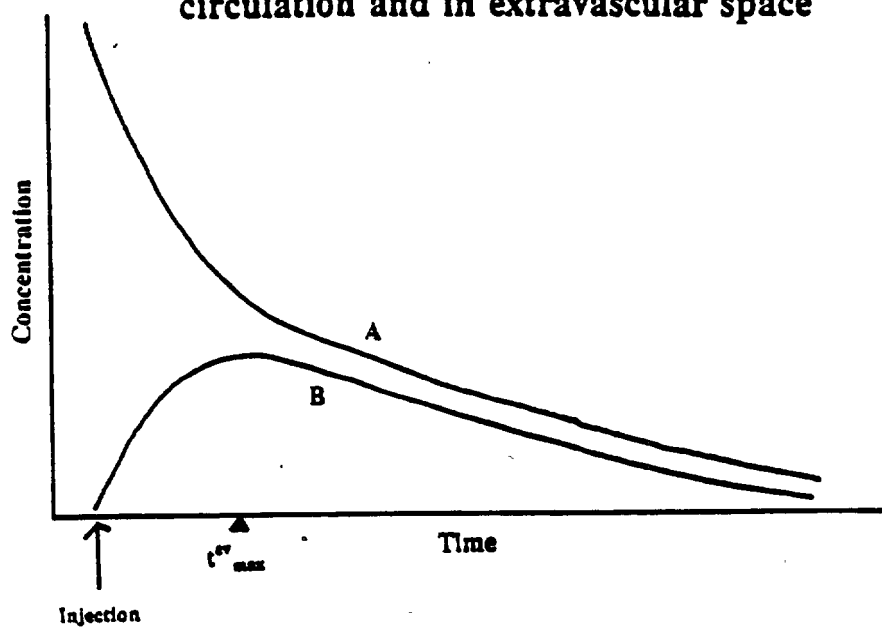
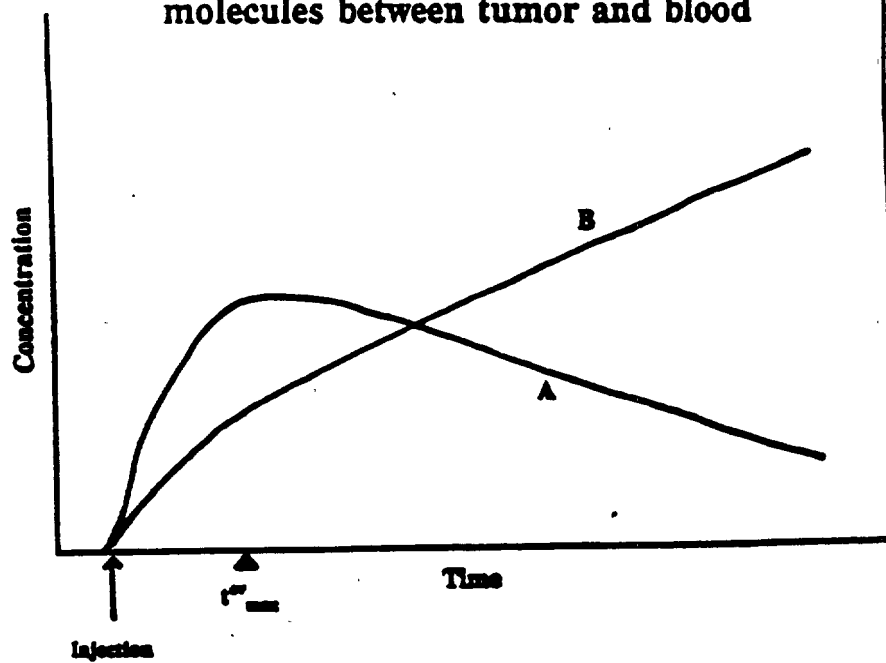
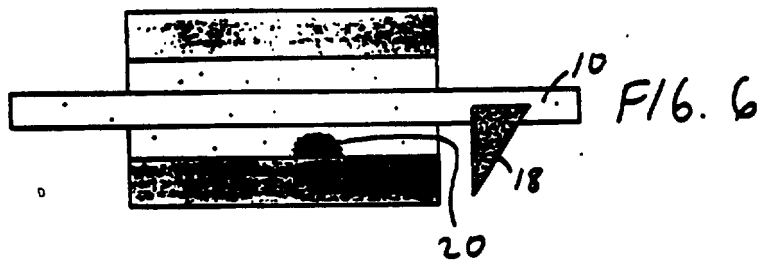
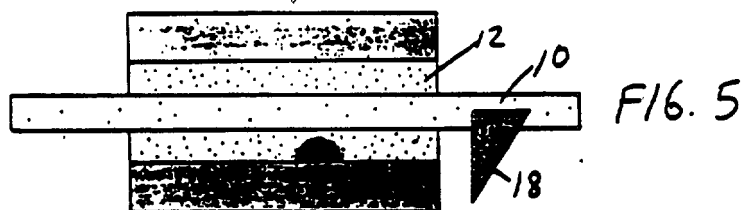
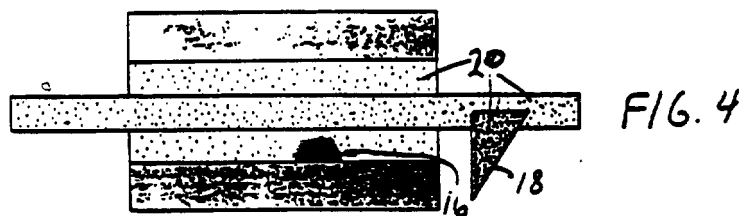
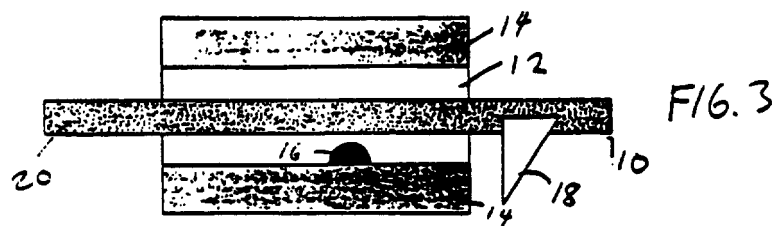


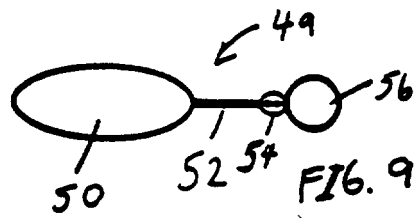
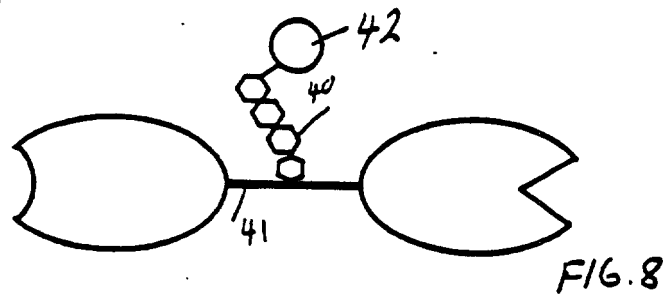
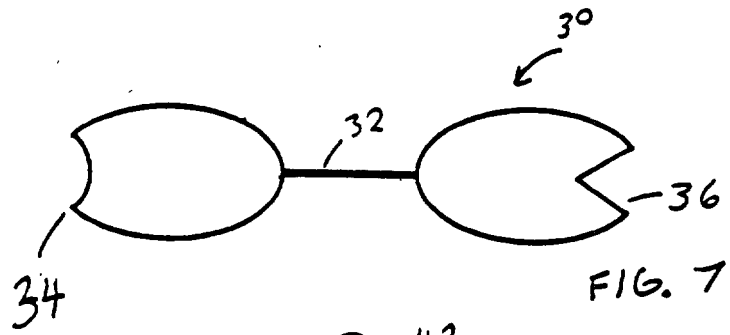
Fig 2

Relative distribution of binding molecules between tumor and blood





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European Patent
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EUROPEAN SEARCH REPORT

Application Number

EP 92 10 5380

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 6)
A, D	US-A-4 946 778 (LADNER R.C. ET AL) * column 11, line 27 - line 35 * * column 30, line 66 - column 31, line 2 * * claims *	1-13	C07K15/00 A61K47/48
A, D	WO-A-8 809 344 (CREATIVE BIOMOLECULES) * page 7, line 8 - line 17 * * page 26, line 8 - page 27, line 19 * * page 52, line 1 - line 30 * * page 67, line 19 - page 68, line 8 *	1-13	
A, P	TRENDS IN BIOTECHNOLOGY, vol. 9, no. 4, April 1991, CAMBRIDGE GB pages 132 - 137; BIRD R.E. ET AL: 'Single chain antibody variable regions.' * the whole document *	1-13	
A	NATURE, vol. 339, no. 6223, 1 June 1989, LONDON GB pages 394 - 397; CHAUDHARY V.K. ET AL: 'A recombinant immunotoxin consisting of two antibody variable domains fused to Pseudomonas exotoxin.' * page 396; figures 1, A, *	1-13	TECHNICAL FIELD SEARCHED (Int. Cl. 6) C07K A61K
A	MEDLINE ABSTRACT Number: 86296741 GIMENEZ-GALLERO G. et al: 'The complete amino acid sequence of human brain-derived acidic fibroblast growth factor.' & BIOCHEM. BIOPHYS. RES. COMMUN., 31 July 1986 138 (2), page 611-617.	1-13	

The present search report has been drawn up for all claims

Place of search BERLIN	Date of completion of the search 28 JUNE 1992	Examiner AMERICAN P.F.
CATEGORY OF CITED DOCUMENTS X: particularly relevant if taken alone Y: particularly relevant if combined with another document of the same category A: technological background D: non-written disclosures P: prior art document T: theory or principle underlying the invention E: earlier patent documents, for published on, or after the filing date B: document cited in the application L: document cited for other reasons A: number of the same patent family, corresponding document		

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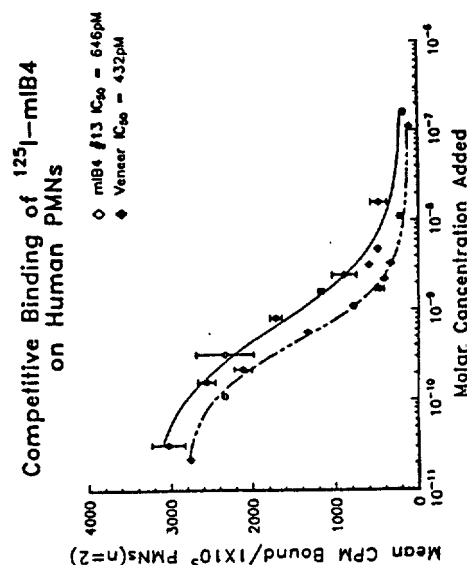
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(54) A method for reducing the immunogenicity of antibody variable domains.

(57) A unique method is disclosed for identifying and replacing immunoglobulin surface amino acid residues which converts the antigenicity of a first mammalian species to that of a second mammalian species. The method will simultaneously change immunogenicity and strictly preserve ligand binding properties. The judicious replacement of exterior amino acid residues has no effect on the ligand binding properties but greatly alters immunogenicity.

Fig. 13



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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Solvent exposure of sidechains of framework residues in KOL and J539 Fvs and the residues which occur most frequently at these positions in the various human VH subgroups.

Figure 2. Solvent exposure of sidechains of framework residues in KOL VL and the residues which occur most frequently at these positions in the various human V-lambda subgroups.

Figure 3. Solvent exposure of sidechains of framework residues in J539 VL and the residues which occur most frequently at these positions in the various human V-kappa subgroups.

Figure 4. Primers used to isolate DNA encoding murine kappa light chain variable region and murine IgG2a heavy chain variable region using PCR. Oligodeoxynucleotides used as PCR primers to generate a shortened IgG4 heavy chain. Oligodeoxynucleotides used in PCR to re-engineer the thymidine kinase (TK) promoter to facilitate the expression of the neomycin resistance gene. Oligodeoxynucleotide primers used in PCR to clone the IgH enhancer sequence. Oligodeoxynucleotides used as PCR primers to generate a human kappa light chain constant region.

Figure 5. Oligodeoxynucleotides used in the construction of the "veneered" 1B4 heavy and light chain variable regions plus those necessary to fuse the human signal and intronic sequences onto these variable regions.

Figure 6. PCR-recombination strategy used in the veneering of the 1B4 kappa light chain variable region.

Figure 7. Outline of the insertion of the "veneered" kappa light chain variable region and kappa constant region into the light chain expression vector.

Figure 8. PCR-recombination strategy used in the veneering of the 1B4 heavy chain variable region.

Figure 9. Outline of the insertion of the "veneered" heavy chain variable region into the heavy chain expression vector.

Figure 10. Outline of the construction of neomycin selectable expression vector.

Figure 11. Outline of the construction of the hygromycin selectable expression vector.

Figure 12. Amino acid sequence completion of the "veneered"-1B4, murine 1B4 and human Gal heavy chain variable regions and the "veneered" 1B4, murine 1B4 and human Len kappa light chain variable regions. Check marks indicate the individual amino acid residues converted.

Figure 13. Competitive binding assay of native murine 1B4 (open diamonds) and recombinant "veneered" 1B4 (closed diamonds).

BACKGROUND OF THE INVENTION

The identification and production of murine monoclonal antibodies has lead to numerous therapeutic applications of these exquisitely specific molecules in human disease. The technologies of molecular biology have further expanded the utility of many antibodies by allowing for the creation of class switched molecules whose functionality has been improved by the acquisition or loss of complement fixation. The size of the bioactive molecule may also be reduced so as to increase the tissue target availability of the antibody by either changing the class from an IgM to an IgG, removing most of the heavy chain constant region in the creation of a F(ab)₂ or both heavy and light chain constant regions may be dispensed with in the formation of a Fv antibody. Common to all of these potentially therapeutic forms of antibody are the requisite CDRs (complementary determining regions) which guide the molecule to its ligand and the framework residues (FRs) which support these latter structures and dictate the disposition of the CDRs relative to one another, Winter European Patent Application, Publication No. 239,400; Riechmann et al., Nature 332: 323-327 (1988). Crystallographic analyses of numerous antibody structures reveal that the combining site is composed almost entirely of the CDR residues arranged in a limited number of loop motifs, Padlan and Sheriff, 1990. The necessity of the CDRs to form these structures combined with the appreciated hypervariability of their primary sequence leads to a great diversity in the antigen combining site, but one which has a finite number of possibilities. Thus, hypermutability and a limited primary sequence repertoire for each CDR would suggest that the CDRs derived for a given antigen from one species of animal would be the same derived from another species. Hence, they should be poorly immunogenic, if at all, when presented to a recipient organism in a non-foreign context.

Monoclonal antibody producing hybridomas have been most readily obtained from immunized rodents. Development of similar reagents from human sources has been frustrated by the current inability to maintain long term cultures of cells which produce sufficient quantities of antibody. Additional problems arise from the regulatory standpoint when cells of human origin are employed for the production of agents to be used in man. These considerations have lead to the widespread use of rodent monoclonal antibodies for the imaging and treatment of malignancy, prophylactic administration to guard against toxic shock, modification of graft rejection episodes, and to temper acute inflammatory reactions. In all scenarios where completely rodent or partially rodent (ie, rodent - human

chimeras) antibodies have been used for therapy the recipients have often elicited an immune response directed toward the antibody. These reactions have limited the duration and effectiveness of the therapy.

Various attempts have been made to minimize or eliminate the immunogenicity of non-human antibodies while perserving their antigen- binding properties. Initially, chimeric antibodies were constructed containing the rodent variable regions and their associated CDRs fused to human constant domains. The following references generally describe chimaeric antibody technology: Lobuglio et al., Proc. Natl. Acad. Sci. USA 86: 4220-4224 (1989); United States Patent 4,816,567; PCT International Publication No. WO 87/02671, published May 7, 1987; European Patent Publication No. 255,694, published February 10 1988; European Patent Publication No. 274,394, published July 13, 1988; European Patent Publication No. 323,806, published July 12, 1989; PCT International Publication No. WO/89/00999, published February 9, 1989; European Patent Publication No. 327,000, published August 9, 1989; European Patent Publication No. 328,404, published August 16, 1989; and European Patent Publication No. 332,424, published September 13, 1989. These proved to be less immunogenic but still approximately half of the recipients mounted an immune response to the rodent variable region framework residues. Further reduction of the "foreign" nature of the chimeric antibodies has been achieved by grafting only the CDRs from the rodent monoclonal into a human supporting framework prior to its subsequent fusion with an appropriate constant domain, Winter European Patent Application, Publication No. 239,400; Riechmann et al., Nature 332: 323-327 (1988). The procedures employed to accomplish CDR-grafting often result in imperfectly "humanized" antibodies. That is to say, the resultant antibody has either lost avidity (usually 2-3 fold, at best) or in an attempt to retain its original avidity a significant number of the murine framework residues have replaced the corresponding ones of the chosen human framework. In this later case, the immunogenicity of the modified "humanized" antibody is difficult to anticipate a priori.

The ligand binding characteristics of an antibody combining site are determined primarily by the structure and relative disposition of the CDRs, although some neighboring residues also have been found to be involved in antigen binding (Davies et al., Ann. Rev. Biochem. 59: 439-473 [1990]). Fine specificity can be perserved in a "humanized" antibody only if the CDR structures, their interaction with each other, and their interaction with the rest of the variable domains are strictly maintained. One may anticipate that the key

residues represent "interior" and interdomain contact residues, hence those surface exposed residues which are immediately available for immune surveillance should be non- inclusive of the structural residues.

OBJECTS OF THE INVENTION

It is, accordingly, an objective of the present invention to provide a means of converting a monoclonal antibody of one mammalian species to a monoclonal antibody of another mammalian species. Another object is to identify the amino acid residues responsible for species specificity or immunogenicity on the exterior of the monoclonal antibody. Another object is judiciously replace or veneer the exterior amino acid residues of one species with those of a second species so that the antibodies of the first species will not be immunogenic in the second species. A further object is to make replacements only in framework regions of the heavy and light chains of the antibody molecule and not in the complementarity-determining regions. Another object of the invention is to provide novel DNA sequences incorporating the replacement amino acid residues. Another object is to provide a vector containing the DNA sequences for the altered antibody. Another object is to provide a eukaryotic or procaryotic host transformed with a vector containing the DNA sequence for the veneered antibody.

SUMMARY OF THE INVENTION

A unique method is disclosed for identifying and replacing immunoglobulin surface amino acid residues which converts the antigenicity of a first mammalian species to that of a second mammalian species. The method will simultaneously change immunogenicity and strictly preserve ligand binding properties. The judicious replacement of exterior amino acid residues has no effect on the ligand binding properties but greatly alters immunogenicity.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a "humanization" procedure which simultaneously reduces the immunogenicity of the rodent monoclonal antibody while perserving its ligand binding properties in their entirety. Since the antigenicity of a protein is primarily dependent on the nature of its surface, the immunogenicity of an xenogenic or allogenic antibody could be reduced by replacing the exposed residues which differ from those usually found in another mammalian species antibodies. This judicious replacement of exterior resi-

dues should have little, or no, effect on the interior domains, or on the interdomain contacts. Thus, ligand binding properties should be unaffected as a consequence of alterations which are limited to the variable region framework residues. The process is referred to as "veneering" since only the outer surface or skin of the antibody is altered, the supporting residues remain undisturbed.

The procedure for "veneering" makes use of the available sequence data for human antibody variable domains compiled by Kabat et al., "Sequences of Proteins of Immunological Interest", 4th ed., Bethesda, Maryland: National Institutes of Health, 1987, updates to this database, and other accessible U.S. and foreign databases (both nucleic acid and protein). The subgroups into which the various sequences have been combined are presented in Figures 1 - 3, indicating the most frequently occurring amino acid at each framework position. Also presented are the sequences of the various J-minigenes. The solvent accessibilities of the amino acids, as deduced from the known three-dimensional structure for human and mouse antibody fragments, are included in these figures.

High resolution X-ray crystallography of the variable domains of the antibodies KOL and J539 have been subjected to extensive refinement beginning with the structures available from the Protein Data Bank (Bernstein et al., J. Mol. Biol. 112: 535-542 1977; file 2FB4 for KOL and file 2FBJ for J529). The solvent accessibilities were computed as described by Padlan Proteins: Struct. Funct. Genet. 7: (1990).

There are two steps in the process of "veneering". First, the framework of a first animal species, i.e. the mouse, variable domains are compared with those corresponding frameworks of a second animal species, i.e. human. It is intended that this invention will allow the antigenic alteration of any animal species antibody. The present invention illustrates the conversion of murine antibody to human antibody, but this is for illustrative purposes only. The most homologous human variable regions are then compared residue for residue to the corresponding murine regions. This will also define the human subgroup to which each mouse sequence most closely resembles. Second, those residues in the mouse framework which differ from its human counterpart are replaced by the residues present in the human counterpart. This switching occurs only with those residues which are at least partially exposed (mE and Ex; Figures 1-3). One retains in the "veneered" mouse antibody: its CDRs, the residues neighboring the CDRs, those residues defined as buried or mostly buried (mB and Bu; Figures 1-3), and those residues believed to be involved with interdomain contacts (boldface, Figures 1-3).

Human and murine sequences frequently differ at the N-terminus of both heavy and light chains. The N-termini are contiguous with the CDR surface and are in position to be involved in ligand binding. Thus, wisdom would dictate that these murine termini be retained in its "veneered" version.

Finally, replacement of some amino acid types could have a significant effect on the tertiary structure or electrostatic interactions of the variable region domains. Hence, care should be exercised in the replacement of proline, glycine, and charged amino acids.

These criteria and the following procedures are used to prepare recombinant DNA sequences which incorporate the CDRs of a first mammalian species, animal, mMAb, both light and heavy chains, into a second mammalian species, human, appearing frameworks that can be used to transfect mammalian cells for the expression of recombinant human antibody with the antigen specificity of the animal monoclonal antibody. The present invention further comprises a method for constructing and expressing the altered antibody comprising: (i) mutagenesis and assembly of variable region domains including CDRs and mutagenesis and assembly of variable region domains including CDRs and FRs regions; (ii) preparation of an expression vector including at least one variable region which upon transfection into cells results in the secretion of protein sufficient for avidity and specificity determinations; and (iii) co-amplification of heavy and light chain expression vectors in appropriate cell lines. The present invention provides recombinant methods for incorporating CDRs from animal monoclonal antibodies into frameworks which appear to be human immunoglobulin in nature so that the resulting recombinant antibody will be either weakly immunogenic or non-immunogenic when administered to humans. Preferably the recombinant immunoglobulins will be recognized as self proteins when administered for therapeutic purposes. This method of "veneering" will render the recombinant antibodies useful as therapeutic agents because they will be either weakly immunogenic or non-immunogenic when administered to humans. The invention is further contemplated to include the recombinant conversion of any animal monoclonal antibody into a recombinant "human-appearing" monoclonal antibody providing that a suitable framework region can be identified (as described below). The animal monoclonals may include, but are not limited to, those murine monoclonal antibodies described by Van Voorhis et al., J. Exp. Med. 158: 126-145 (1983) which bind to human leukocytes and the appropriate mMAbs produced by hybridomas deposited in the Hybridoma Cell Bank maintained by the American Type Culture Collection (ATCC) and

described in the ATCC Catalog of Cell Lines 8 Hybridomas, No. 6, 1988.

The CDR sequences from the animal monoclonal antibody are derived as follows. Total RNA is extracted from the murine hybridomas, for example the 1B4 myeloma cells described by Wright et al., Proc. Natl. Acad. Sci. USA 80: 5699-5703 (1983), the 60.3 cells described by Beatty et al., J. Immunol. 131:2913-2918 (1983), the TS1/18 cells described by Sanchez-Madrid et al., J. Exp. Med. 158: 1785-1803 (1983), and other anti-CD18 or CD11 monoclonal antibodies and hybridomas as described in Leukocyte Typing III, Springer-Verlag, New York (1988), using standard methods involving cellular solubilization with guanidinium isothiocyanate (Chirgwin et al., Biochem. 18: 5294-5299 [1979]). The murine 1B4 mAb will be used as the primary example of animal MAb that can be "veneered" by the unique process being disclosed. The invention is intended to include the conversion of any animal immunoglobulin to a "human-appearing" immunoglobulin. It is further intended that "human-appearing" immunoglobulin (Ig) can contain either kappa or lambda light chains or be one of any of the following heavy chain isotypes (alpha, delta, epsilon, gamma and mu).

Pairs of degenerate oligodeoxynucleotide primers (Figure 4) representing sequences within framework 1 of the murine kappa light chain variable region and light chain constant domain, or those within framework 1 of the murine IgG2a heavy chain variable region and heavy chain constant CH1 domain are synthesized on an Applied Biosystem 381A DNA synthesizer, removed from the resin by treatment with concentrated NH_4OH and desalted on a NAP-5 column eluted with H_2O . Total RNA, about 2 μg , is reverse transcribed for 30 min at 42°C using Moloney MLV reverse transcriptase, about 200 units (BRL), and about 10 pmoles of the constant region complementary strand primers for either the heavy or light chain. The reverse transcriptase is heat inactivated, about 95°C for about 5 min, and the reactions are made to contain in about 100 μl of PCR buffer about 50 pmoles of each of the paired primers and 2.5 units of Taq polymerase. About 45 cycles of amplification ($2'$, 94°C ; $2'$, 55°C ; $2'$, 72°C) are followed by gel purification of the anticipated 400 + base pair (bp) DNA fragments. Prior to subcloning those DNAs into a blunt-ended intermediate plasmid such as pSP72 (Promega) they are terminally phosphorylated using T4 polynucleotide kinase. Multiple clones representing these PCR amplified sequences are grown and submitted to DNA sequence determinations using Sequenase® and T7 and SP6 specific sequencing primers. A unique DNA sequence representing a murine IgG2a heavy chain variable region is obtained by analysis of the

derived amino acid sequences. Replacement of the "murine-appearing" framework residues with those residues compatible with a human variable region is accomplished utilizing the following unique processes. An appropriate human framework is determined utilizing the criteria discussed below. The light chain variable region framework with sufficient homology to the the m1B4 framework was determined to be the human LEN framework (FR). The Len FR shows a similarity of 90% and an identity of 81% when compared to murine 1B4. This sequence, with its leader, 3' intronic sequences and engrafted m1B4 CDRs had been subcloned into the intermediate vector pGEM3Z (Promega), as described in Daugherty et al. Nucleic Acids Res. 19: (1991). About eight oligodeoxynucleotide primers (Figure 5) are synthesized representing the primers necessary to generate by polymerase chain reaction (PCR) amplification four DNA fragments. Incorporated into all but the terminal oligodeoxynucleotide primers were those sequences corresponding to the veneered MAb 1B4 light chain, with its unaltered CDRs, and at least 15 bases of 5'-terminal complementarity to allow for the subsequent PCR- directed recombination of these four fragments. For the purposes of exemplifying the "veneering" process the LEN light chain variable region already containing an engrafted set of CDRs representing those within the light chain of murine 1B4 was used as the template into which mutations were placed so as to easily create the "veneered" framework sequence. The appropriate primer pair (S1 & V9, V10 & V11, etc.), about 50 pmole each, was combined with about 10 ng of plasmid DNA representing the LEN CDR-grafted framework, about 2.5 units of Taq DNA polymerase and about twenty-five (25) cycles of PCR amplification ensued (cycle periods: $1'$, 94°C ; $1'$, 55°C ; $2'$, 72°C). The products of the four reactions, purified by agarose gel electrophoresis, are combined, about 10 ng of each DNA fragment, along with terminal oligodeoxynucleotide primers (A1 & A2, Figure 6) and Taq DNA polymerase. The combined fragments were PCR amplified (25 cycles of: $2'$, 94°C ; $2'$, 55°C ; $2'$, 72°C). Following restriction endonuclease digestion with Hind III and Xba I the amplified DNA is purified by agarose gel electrophoresis and subcloned into compatible sites of an intermediate vector pSP72 (Promega) which contains the human kappa light chain constant region (see Figure 7). Genomic DNA, about 1 μg , purified from a human B cell line (GM0108A: NIGMS Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, NJ) is used as a template for PCR amplification (Figure 4) of about a 920 base pair fragment containing the splice acceptor for the kappa light chain constant domain, the exon and a portion of its 3'-untran-

slated region. The PCR product is purified by agarose gel electrophoresis, digested with Bam H1 endonuclease, and subcloned into pSP72 previously linearized with Bam H1. The individual clones representing the pSP72 intermediate vector containing both the 1B4 "veneered" light chain variable region and the human kappa constant region derived by PCR amplification of human DNA are used to determine the DNA sequence of the "veneered" light chain variable region.

The "veneered" heavy chain portion of the recombinant antibody is derived from the mutated version of the murine 1B4 heavy chain variable region fused to the human constant region of a gamma 4 subtype obtained from a lambda library constructed by Flanagan and Rabbits, *Nature* 300: 709-713 (1982). The variable region of the "veneered" heavy chain is constructed from five DNA fragments representing a signal sequence, portions of the mutated murine heavy chain variable region, and an intronic sequence (Figure 8). Oligodeoxynucleotide primer pairs (Figure 5) are synthesized representing the primers necessary to generate by PCR amplification these five DNA fragments from about 10 ng of plasmid DNA template obtained from a pSP72 intermediate vector containing the heavy chain variable region previously used to determine the murine 1B4 CDR sequence. Amplification of the signal fragment, variable region fragments, and intron-containing fragment was as described above. The agarose gel purified products are combined, about 10 ng of each product, with terminal oligodeoxynucleotide primer pairs (Figure 8) and the PCR-generated in vitro recombined template is amplified using the standard procedures described above. Prior to subcloning into a Hind III and Bam HI digested expression vector containing the human heavy chain gamma 4 constant region (Figure 9), this recombined product is similarly digested and agarose gel purified. Individual clones are submitted to DNA sequence determination using Sequenase® and T7 and SP6 specific sequencing primers and one is chosen for subsequent expression. The gamma 4 heavy chain constant region is subcloned as about a 6.7 Kb Hind III fragment derived from the plasmid pAT84 into the Hind III site of the intermediate vector pSP72. This plasmid is then used as the template DNA from which a shortened version of the gamma 4 constant region is subcloned using PCR amplification and the primer pairs indicated in Figure 4. Eukaryotic expression vectors are constructed as described below.

Expression vectors are defined herein as DNA sequences that are required for the transcription of cloned copies of genes and the translation of their mRNAs in an appropriate host. Such vectors can be used to express eukaryotic genes in a variety of

hosts such as bacteria, blue-green algae, plant cells, yeast cells, insect cells and animal cells. The immunoglobulins may also be expressed in a number of virus systems. Specifically designed vectors allow the shuttling of DNA between host such as bacteria-yeast or bacteria-animal cells. An appropriately constructed expression vector should contain: an origin of replication for autonomous replication in host cells, selectable markers, a limited number of useful restriction enzyme sites, a potential for high copy number, and strong promoters. A promoter is defined as a DNA sequence that directs RNA polymerase to bind to DNA and initiate RNA synthesis. A strong promoter is one which causes mRNAs to be initiated at high frequency. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses. The heavy chain immunoglobulin molecule is transcribed from a plasmid carrying the neomycin (G418) resistance marker while the light chain immunoglobulin is transcribed from a plasmid carrying the hygromycin B resistance marker. With the exception of the drug resistance portion of these plasmids they are identical. The preferred progenitor of the immunoglobulin expression vectors is the pD5 (Berkner and Sharp, *Nucl. Acids Res.* 13: 841-857 [1985]) eukaryotic expression vector which contains the origin of adenovirus replication, the SV40 enhancer domain, the adenovirus major late promoter, the adenovirus 2 tripartite leader, a 5' splice donor from the adenovirus third leader and a 3' splice acceptor derived from an immunoglobulin locus, a multiple cloning site placed in the Bam H1 site subsequent to receipt of the vector, and the SV40 late polyadenylation signal (Figure 10). The origin of replication is removed by digestion with Eco RI and Kpn I and replaced by two fragments representing the neo selectable marker gene (derived from plasmid pCMVIE-AK1-DHFR as an Eco RI/Bam H1 about 1.8 Kb fragment) and the Ig heavy chain enhancer (obtained as a PCR amplified fragment using human DNA as the template, and the oligodeoxynucleotides listed in Figure 4 as the primer pair, following its digestion with Bgl II and Kpn I). The resultant expression vector is found to lack a small portion of the TK promoter responsible for the transcription of the neomycin gene. This is replaced by insertion into the Eco RI site about a 0.14 Kb PCR amplified fragment derived from the CMVIE-AK1-DHFR DNA using the primer pair listed in Figure 4. The resultant heavy chain expression vector (p8941) is modified by removal of the indicated Hind III and Xba I sites using standard procedures. To convert this vector into one expressing the hygromycin B selectable marker the neomycin-resistance cassette is removed by digestion first with Eco RI followed by

DNA polymerase-directed fill in of the 5' overhand, then subsequent Sal I digestion. The about 1.9 Kb hygromycin B expression cassette, TK promoter and TK polyadenylation signal flanking the hygromycin B gene, (obtained as a 1.8 kb Bam HI fragment in plasmid pL690, Gritz and Davies, Gene 25: 179-188 [1981]) is removed from the plasmid pAL-2 by Bam HI digestion and subcloned into the Bam HI site of the intermediate vector pSP72. The hygromycin B cassette is removed from this vector by digestion with Sma I and Sal I and cloned into the expression vector linearized as described above to create a blunt end and Sal I end DNA fragment (Figure 11).

Expression of the 1B4 "veneered" kappa light chain is accomplished by transferring this cistron from the pSP72-based intermediate cloning vector (p8952), containing the human kappa constant region, to the hygromycin B selectable eukaryotic expression vector (Figure 7). An about 1.5 kb DNA fragment resulting from the endonuclease digestion of p8952 with Spe I and Cla I is purified by agarose gel electrophoresis and ligated into the expression vector which has previously been linearized, following digestion with the same two restriction enzymes, and agarose gel purified. The heavy chain eukaryotic expression vector is constructed in two steps. First, the p8950 vector containing the modified heavy chain variable region of murine 1B4 fragment is digested with Bgl II and Bam HI. The agarose gel purified 0.75 kb fragment is ligated into the Bam HI site of the p8941 vector and recombinant clones containing this fragment in the proper orientation are identified. Plasmid DNA from one such clone is linearized by Bam HI digestion and ligated with a 1.78 Kb Bam HI fragment representing a short version of the human gamma 4 constant region, derived from plasmid pAT84 by PCR amplification. Following the identification of clones containing these inserts in the appropriate orientation, plasmid DNAs (one which is referred to as p8953) are grown and purified for transfection into recipient mammalian cells. Equal amounts, about 10 µg, of the plasmids encoding the 1B4 "veneered" IgG4 heavy chain and the 1B4 "veneered" kappa light chain are transfected by standard calcium phosphate precipitation procedures into the monkey kidney cell line CV-1P or the human embryonic kidney cell line 293. The culture supernants, assayed by a trapping ELISA (described below), were found to contain a human kappa light chain / human IgG4 immuno-globulin. Immulon-2 (Dynatech Labs.) 96-well plates are coated overnight with about a 5 µg/ml solution of mouse anti-human kappa chain constant domain monoclonal antibody (cat. #MC009, The Binding Site, Inc., San Diego, CA) in about 0.1 M NaHCO₃ buffer (pH 8.2) at about 4° C, and blocked with

about 1% bovine serum (BSA) in about 0.1 M NaHCO₃ for about 1 hour at about 25° C. After this and all subsequent steps, washing was performed with phosphate buffered saline (PBS). The wells are then challenged with conditioned medium containing recombinant anti-CD18 antibody, or with predetermined quantities of human IgG4/kappa purified by protein A Sepharose (Pharmacia Fine Chemicals) chromatography from human IgG4 myeloma serum (cat. # BP026, The Binding Site, Inc.) All samples are diluted in PBS containing about 0.05% Tween-20. About 100 µl aliquots are incubated for about 1 hour at about 37° C in triplicate, and standard calibration curves are constructed using IgG4 concentrations ranging from about 10 ng/ml to about 100 ng/ml. Bound and fully assembled human IgG4 (either native or the recombinant 1B4 human "veneered" IgG4 constructs) are detected with about 100 µl aliquots of a 1:500 dilution of mouse anti-human IgG4 Fc monoclonal antibody conjugated to alkaline phosphatase (cat #05-3822, Zymed Laboratories, Inc.) in phosphate buffered saline (PBS) containing about 1 % BSA. After incubation for about 1 hour at about 37° C and subsequent washing, the quantities of bound conjugate are detected by incubating all samples with a 1 mg/ml solution of p-nitrophenyl phosphate in 0.1 M 2,2' amino methylpropanediol buffer, pH 10.3, for about 30 minutes at about 25° C. The adsorbance of the wells is determined with a UV Max ELISA plate reader (Molecular Devices) set at 405 nm. The antibody secreted by the transfected human 293 cells or monkey kidney CV1 P cells, either following transient expression or subsequent to stable clone isolation, is isolated by protein A chromatography, the concentration of recombinant human anti-CD18 antibodies determined by the trapping Elisa described above, and used to compete with the binding of radiolabeled murine 1B4 to the CD18 ligand on the surface of activated human PMNs. Affinities of r-anti-CD18 antibody constructs are determined using a competitive ¹²⁵I-1B4 soluble binding assay with stimulated human polymorpho-nuclear leukocytes (PMNs). Purified murine anti-CD18 monoclonal antibody (50 µg) is iodinated using chloramine-T (Hunter, W.M. and Greenwood, F.C., Nature 194: 495-496, 1962), and the radiolabeled antibody purified using a Bio-Sil TSK250 (Biorad, Richmond, CA) gel filtration HPLC column (which fractionates proteins in the range of 1-300 x 103 daltons) equilibrated in 0.1 M phosphate buffer, pH 7.0. Effluent radioactivity is monitored with an inline detector (Beckman Model 170; Beckman, Fullerton, CA) and total protein measured at OD₂₈₀ with a Kratos Spectroflow 757 detector (Kratos, Mahwah, N.J.). A single ¹²⁵I-1B4 peak composed of coincident OD₂₈₀ and radioactivity tracings char-

acteristically elutes at about 6 minutes, 30 seconds following sample injection. Specific activity of the product is generally about 10 $\mu\text{Ci}/\mu\text{g}$ protein, and 97-99% of the counts are precipitable with 10% trichloroacetic acid. The binding of this radiolabeled antibody is assessed on human PMNs purified on a discontinuous Ficoll/Hypaque gradient (English, D. and Anderson, B.R., J. Immunol. Methods 5: 249-255, 1974) and activated with about 100 ng/ml phorbol myristate acetate for about 20 minutes at about 37°C (Lo et al., J. Exp. Med. 169: 1779-1793, 1989). To determine the avidity of antibodies for CD18 molecules on the PMN surface, about 1×10^5 activated PMNs are incubated in a buffer such as Hanks balanced salt solution containing about 20 mM Hepes (pH 7.2), about 0.14 units aprotinin (Sigma Chemical Co.) and about 2% human serum albumin (binding buffer) containing about 1.3 ng ^{125}I -1B4 (2.8×10^{-11} M) in the presence of increasing concentrations of unlabeled 1B4 antibody (about 10^{-7} to 10^{-15} M) in about a 300 μl reaction volume for about 1 hour at about 4°C with constant agitation. Cell bound 1B4 is separated from the unbound antibody by centrifugation through a 0.5 M sucrose cushion (4,800 x g, 3 minutes); the tubes are frozen on dry ice, and the tips cut off and counted with an LKB gamma counter. The IC_{50} of the anti-CD18 antibody for the inhibition of ^{125}I -1B4 antibody binding is calculated using a four parameter fitter program (Rodbard et al., In, "Radioimmunoassay and Related Procedures in Medicine", International Atomic Energy Agency, Vienna, vol 1,469 - 504, 1978). The affinity of the "veneered" r-anti-CD18 antibody for the CD18 ligand is determined in a similar manner using murine ^{125}I -1B4 antibody and increasing quantities, as determined by the trapping Elisa, of unlabeled r-anti-CD18. The results of the binding assays are shown in Figure 13 and indicate that the avidity of the "veneered" recombinant 1B4 antibody is equal to that of the murine 1B4 monoclonal antibody. This result shows that an antibody with presumptive human isotype may be recombinantly constructed from the murine parent antibody by the introduction of numerous point mutations in its framework residues and expressed fused to human kappa and gamma 4 constant domains without loss in avidity for the antigen. It can be inferred from this result that the point mutations within the framework regions do not alter the presentation of the murine 1B4 light chain and heavy chain CDRs. Many of the examples of construction of recombinant human antibodies containing complementarity regions replaced by those found within murine monoclonal antibodies have resulted in loss of avidity for the ligand or antigen. Thus, although these latter transmutations are possible, the successful maintenance of avidity is not assured. This

procedure described above demonstrates that when strict attention is paid to the framework regions, and the nature of the amino acids within each framework, "humanization" may potentially be achieved without the loss of avidity which accompanies the transfer of CDRs to the "generic" human frameworks ("humanization") employed by Winter, European Patent Publication No. 239,400, published September 30, 1987.

To identify human framework sequences compatible with the CDRs of, say, murine 1B4, human frameworks with a high degree of sequence similarity to those of murine 1B4 are identified. Sequence similarity is measured using identical residues as well as evolutionarily conservative amino acid substitutions. Similarity searches are performed using the murine 1B4 framework sequence from which the CDR sequences had been removed. This sequence is used to query a database of human immunoglobulin sequences that had been derived from multiple sources. Sequences with a high degree of sequence similarity are examined individually for their potential as humanizing framework sequences. In this way, the human homologue providing the murine CDRs with the structure most similar to their native murine framework is selected as the template for the construction of the "veneered" variable regions (Figure 12). Should human frameworks of sufficient similarity not be identifiable from compiled sequences, it is possible to isolate from human genomic DNA a group of closely related variable regions using recombinant technology. Thus, a degenerate 5' upstream oligodeoxynucleotide primer may be designed from the conserved sequences within the amino-terminus of each of the various human FR1 regions and paired with a degenerate 3' downstream oligodeoxynucleotide primers fashioned from the FR sequence determined from the murine monoclonal whose CDRs one wishes to transfer into a human context. These primer pairs are then used to PCR amplify from a human genomic template those DNA sequences which are flanked by the primer pair. The resulting DNAs may then be cloned and the DNA sequence derived from individual members will describe various murine-related human variable regions. The paucity of somatic mutations in framework residues and the conservation of amino acid sequence between mouse and man make this approach possible.

The construction of a complete recombinant human IgG4 antibody, whose heavy and light chain variable domains contain the CDR residues of the murine monoclonal antibody, with complete retention of the specificity and avidity of the parent murine monoclonal antibody is disclosed. The construction of the "veneered" light chain framework derived from the human sequence of LEN fused

with a human kappa light chain constant region is described above. The murine variable region framework sequence, devoid of CDR sequences, is used to query a database of complete human variable region sequences. The human sequences that are most similar to the murine framework region are then analyzed individually to determine both their sequence identity and similarity to the murine framework region. In the case of murine 1B4 these sequences include, but are not limited to, "Gal", chosen because of its high degree of both similarity and identity with the 1B4 heavy chain sequence. The Gal FR has been found to be 85% similar and 79% identical to murine 1B4. These values are based upon the Dayhoff similarity matrix of evolutionarily conserved amino acid substitutions (R. M. Schwartz, M. O. Dayhoff, in Atlas of Protein sequence and structure M. O. Dayhoff, Eds. (National Biomedical Research Foundation, Washington, DC [1979]) (Figure 12). To prepare a recombinant DNA encoding the murine heavy chain CDRs in the context of a human-appearing framework the following procedures are performed. A set of ten short oligodeoxynucleotides are synthesized. Each pair is combined in a separate PCR reaction with the DNA template representing the murine 1B4 heavy chain variable region, amplified and isolated following PCR of the RNA of the murine hybridoma 1B4 as described above. Thus, about 50 pmole of each primer pair was combined with about 10 ng of plasmid DNA representing the murine 1B4 heavy chain variable region, about 2.5 units of Taq DNA polymerase and about twenty-five (25) cycles of PCR amplification ensued (cycle periods: 1', 94°C; 1', 55°C; 2' 72°C). The products of the five reactions (Figure 8) encoded portions of the 1B4 heavy chain variable region, beginning with the signal peptide encoding region and ending with the 3' intronic sequence which resides between the variable region coding domain and the IgG4 constant region sequence, with the desired point mutations to create a "veneered" variable region framework. These five fragments are purified by agarose gel electrophoresis, combined, about 10 ng of each DNA fragment, along with terminal oligodeoxynucleotide primers (A1 & A2, Figure 5) and Taq DNA polymerase. The combined fragments were PCR amplified (25 cycles of: 2', 94°C; 2', 55°C; 2' 72°C). By virtue of the complementary ends of the five fragments, the polymerization/denaturation/polymerization cycles of the polymerase chain reaction result in the formation, and subsequent amplification, of the combined sequences. Following 25 cycles of amplification the combined 0.8 Kb fragment is electrophoretically purified from an agarose gel and was digested with restriction enzymes Spe I and Bam H1. Following agarose gel electrophoresis, the

purified DNA fragment is ligated into the heavy chain expression vector, p8958 (see Figure 9), in place of the chimaeric variable region existing in this vector. Each "veneered" variable region, with its associated human constant region, residing within a pD5-based expression vector plasmid was co-transfected into 293 cells and CV1 P cells and recombinant human antibody is found to be present in the conditioned medium 48 hours post transfection. The "veneered" recombinant antibody is isolated by protein A chromatography. The avidity of this antibody for the CD18 ligand displayed on the surface of activated human PMNs is compared with that of the murine 1B4 monoclonal antibody parent. Figure 13 shows that although each antibody contains the same set of six CDRs within different framework domains, they exhibit identical avidity for the ligand. Thus, the avidity of an antibody molecule does not rely upon the variable region framework residues which are surface exposed, rather the proper structure in which the CDRs are presented must be significantly influenced by the buried and inter/intra active residues. The parent murine monoclonal antibody demonstrates an IC_{50} of about 1.0 to about 0.7 nM, the "veneered" molecule has a similar IC_{50} .

This invention further relates to a method of inhibiting the influx or migration of leukocytes capable of expressing CD18 antigen (leukocyte integrin, beta subunit) on their surface into a site of inflammation or a tissue area or organ that will become inflamed following an influx of the cells. The inflammation which is the target of the method of the present invention may result from an infection with pathogenic microorganisms such as gram-positive and gram-negative bacteria, parasites and fungi. The response may also be induced by viruses and non-infectious means such as trauma or reperfusion following myocardial infarction or stroke, immune responses to foreign antigen and autoimmune responses. The recombinant human anti-CD18 antibodies are useful in the treatment of inflammation in lung, central nervous system, kidney, joints, endocardium, eyes, ears, skin, gastrointestinal tract and urogenital system. Disease states in which the recombinant human anti-CD18 antibodies are useful as therapeutic agents include, but are not limited to: infectious diseases where active infection exists at anybody site, such as meningitis; conditions such as chronic or acute secondary inflammations caused by antigen deposition; and other conditions such as, encephalitis; arthritis; uveitis; colitis; glomerulonephritis; dermatitis; psoriasis; and respiratory distress syndrome associated with sepsis and/or trauma. Other inflammatory diseases which may be responsive to recombinant human anti-CD18 antibody include, but are not limited to, immune disorders and conditions involv-

ing T-cell and/or macrophage attachment/recognition, such as acute and delayed hypersensitivity, graft vs. host disease; primary autoimmune conditions such as pernicious anemia; infection related autoimmune conditions such as Type I diabetes mellitus; flares during rheumatoid arthritis; diseases that involve leukocyte diapedesis, such as multiple sclerosis; antigen-antibody complex mediated diseases including certain of the secondary infection states listed above; immunosuppression; and transplant rejection. Inflammatory conditions due to toxic shock or trauma such as adult respiratory distress syndrome and reperfusion injury; and disease states due to leukocyte dyscrasias and metastasis, are included within the scope of this invention. The present invention is also applicable to the inhibition of leukocyteendothelial attachment for diagnostic and therapeutic purposes; such as the iatrogenic opening of the endothelium to prevent the ingress of leukocytes during the ingress of a therapeutic drug in the instance of chemotherapy; or to enhance the harvesting of leukocytes from patients.

Recombinant human anti-CD18 antibodies or an active fragment thereof can be used to treat the above mentioned diseases. An active fragment will include the F(ab')₂, the Fab and any other fragment that can bind to the CD18 antigen. Recombinant human anti-CD18 antibodies can be administered alone for non-infectious disease states or combined with antibiotics or other anti-infective agents for the treatment of infectious diseases for reasons discussed above. Administration will generally include the antibodies and possibly other substances in a physiologically acceptable medium or pharmaceutical carrier. Such physiologically acceptable media or pharmaceutical carriers include, but are not limited to, physiological saline, phosphate buffered saline, phosphate buffered saline glucose, buffered saline and the like. The antibodies and any anti-infective agent will be administered by parenteral routes which include intravenous, intramuscular, subcutaneous and intraperitoneal injection or delivery. The amount of the antibodies and the mixture in the dosage form is dependent upon the particular disease state being treated. The amount of the recombinant human anti-CD18 antibody utilized in a dosage form can range from about 1 to about 1,000 mg, with a range of from about 10 mg to about 100 mg being preferred. The antibodies can be administered daily or less than daily as determined by the treating physician. The following examples illustrate the present invention without, however, limiting the same thereto.

EXAMPLE

Preparation of a "Veneered" Recombinant Antibody

An antibody was produced in which the variable domain of the light chain comprises the framework region of a murine light chain modified to contain surface exposed amino acids of human derivation. The variable domain of the heavy chain is similarly derived from the murine heavy chain with point mutations which replace murine exposed residues with human-appearing residues. The light chain human framework region was derived from human myeloma protein LEN. The CDR and framework sequences from the murine monoclonal antibody 1B4 which binds to CD18 (the beta subunit of the leukocyte integrin B-2 family which includes: LFA-1, Mac-1, and p150.95) were derived as follows. The hybridoma designated 1B4 which produces 1B4 monoclonal antibody was deposited under the Budapest Treaty at the International Depository Authority: American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, 20852. Viability was determined on June 6, 1989 and the hybridoma was designated HB 10164. Previous experiments had determined this antibody to be an IgG 2a with a kappa light chain (Wright et al., Proc. Natl. Acad. Sci. USA 80: 5699-5703 [1983]).

Total RNA was extracted from the 1B4 myeloma cells using standard methods involving cellular solubilization with guanidinium isothiocyanate (Chirgwin et al., Biochem. 18:5294-5299 [1979]). Sets of degenerate oligodeoxynucleotide primers (Figure 4) representing sequences within framework 1 of the murine kappa light chain variable region and kappa light chain constant domain, or those within framework 1 of the murine IgG2a heavy chain variable region and heavy chain constant CH1 domain were synthesized by standard phosphoramidite procedures on an Applied Biosystem 381A DNA synthesizer. Removal of the oligodeoxy-nucleotides (oligos) from the resin was accomplished by treatment with concentrated NH₄OH followed by desalting on a NAP-5 column (Pharmacia) with H₂O elution (when the oligos were <45 bases in length), or by use of an OPC column (Applied Biosystems Inc) with 20% acetonitrile elution (when the oligos were >45 bases in length), as recommended by the manufacturers. Total RNA (2μg) was reversed transcribed for 30' at 42°C using Moloney MLV reverse transcriptase (200 units, BRL) and 10 pmoles of the constant region complementary strand primers representing either heavy or light chain in a buffer (final volume of 20 μl) containing 50 mM Tris HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, and 20 units of RNAsin (Pharmacia). The reverse transcriptase was heat inactivated (95°C, 5') and the reactions were made to contain in 100μl of

PCR buffer (10 mM Tris HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 200 μ M each dNTP), 50 pmoles of each of the paired primers, and 2.5 units of Taq polymerase (Perkin Elmer/Cetus). Polymerase chain reaction (PCR) amplification was carried out essentially as described by Saiki et al., *Science* 230: 1350-1354 (1985) and others (Mullis et al., *Cold Spring Harbor Symp. Quant. Biol.* 51: 263-273 [1986], Dawasaki and Wang, *PCR Technology, Principles and Applications for DNA Amplification*, Erlich, Ed., Stockton Press, NY, pp. 89-97 [1989], Tung et al., *ibid.* pp. 99-104 [1989]). Forty five cycles of amplification by a DNA Thermal Cycler (Perkin Elmer Cetus Instruments) (2', 94°C; 2', 55°C; 2' 72°C) were followed by gel purification of the anticipated 400+ base pair (bp) DNA fragments. Prior to subcloning the DNAs into a blunt-ended intermediate plasmid (pSP72, Promega) they were terminally phosphorylated using T4 polynucleotide kinase (Boehringer Mannheim). Multiple clones representing these PCR amplified sequences were isolated from DH5 transformed E.coli plated on LB agar plates containing 50 μ g/ml ampicillin, grown by described procedures (Maniatis et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982), plasmid DNAs were extracted from the bacteria using the DNA preparation procedures of Birnboim and Doly, *Nucleic Acid Res.* 7: 1515 (1979), and the double-stranded plasmid DNAs were submitted to DNA sequence determinations using Sequenase® (United States Biochemicals) and T7 and SP6 specific sequencing primers (Boehringer Mannheim) using the protocols recommended by the manufacturer. A unique DNA sequence representing a murine IgG2a heavy chain variable region was obtained, as was a kappa light chain variable region sequence.

To give the final appearance of a "veneered" murine light chain, several residues within a template composed of the human LEN framework, into which had been grafted the CDRs described for 1B4, were replaced by corresponding residues found in the murine 1B4 light chain framework. Replacement of the human LEN variable region residues with those unique to MAb 1B4 took place as follows. Eight oligodeoxynucleotides (Figure 5) were synthesized representing the primers necessary to generate by PCR amplification four DNA fragments. Incorporated into all but the terminal oligodeoxynucleotides were those sequences corresponding to the MAb 1B4 light chain variable region framework residues to be point mutated and at least 15 bases of 5'-terminal complementarity (see Figure 6). The appropriate primer pair (50 pmole each) was combined with 10 ng of a 1B4 CDR-grafted LEN framework-containing plasmid

DNA, 2.5 units of Taq DNA polymerase, PCR reaction components and buffer, and twenty-five (25) cycles of PCR amplification ensued (cycle periods: 1', 94°C; 1', 55°C; 2' 72°C). The products of the four reactions, purified by agarose gel electrophoresis, were combined (10 ng of each DNA fragment) along with a terminal oligodeoxynucleotide primer pair (amplifier) (Figures 5 & 6), Taq DNA polymerase, PCR reaction components and buffer, and the subsequent recombined fragments were amplified, as described above, for twenty-five (see Figure 6). Following restriction endonuclease digestion with HindIII and XbaI the amplified DNA was purified from an agarose gel and subcloned into these same sites of an intermediate vector pSP72 (Promega) which contained the human kappa light chain constant region, obtained as follows. DNA (1 μ g) purified from a human B cell line (GM01018A; NIGMS Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, N.J. 08103) was used as a template for the oligodeoxynucleotide primers described in Figure 4 to PCR amplify a 920 base pair fragment containing the splice acceptor for the human kappa light chain constant domain, the exon and a portion of its 3'-untranslated region (PCR primer pair choice was selected based on the kappa constant region sequence described by Hieter et al., *Cell* 22: 197-207 (1980). The PCR product was purified by agarose gel electrophoresis, digested with Bam H1 endonuclease, and subcloned into pSP72 (Promega) previously linearized with Bam H1.

The individual clones representing the pSP72 intermediate vector containing both the 1B4 "veneered" light chain variable region derived as described above, and the human kappa constant region, derived by PCR amplification of human DNA, were used to verify the DNA sequence of the "veneered" light chain variable region. The "veneered" heavy chain portion of the recombinant antibody was derived from a point mutated murine 1B4 heavy chain variable region fused to the human constant region of gamma 4 subtype obtained from a lambda library constructed by Flanagan and Rebbitts, *Nature* 300: 709-713 (1982).

The variable region of the "veneered" heavy chain was constructed from five DNA fragments representing a signal sequence, mutated portions of the murine 1B4 heavy chain variable region, and an intronic sequence (Figure 8). Oligodeoxynucleotide primer pairs (Figure 5) were synthesized representing the primers necessary to generate by PCR amplification these five DNA fragments from 10 ng of plasmid DNA template containing the murine 1B4 heavy chain variable region previously used to determine the murine 1B4 CDR and framework sequences. Amplification of the five fragments was performed as described above for the four

light chain variable region fragments. The agarose gel purified products were combined (10 ng of each product) with terminal primer pairs (Figure 5) and the PCR-generated *in vitro* recombined template was amplified using the standard procedure also described above for recombining the fragments comprising the "veneered" light chain variable region. Prior to subcloning into a Hind III and Bam HI digested expression vector this recombinant product was similarly digested and agarose gel purified. DNA was obtained following growth of individual bacterial clones and submitted to DNA sequence determination using Sequenase® and T7 and SP6 specific sequencing primers in order to verify the sequence of the reconstructed variable region and its flanking domains.

The gamma 4 heavy chain constant region had been subcloned as a 6.7 Kb Hind III fragment derived from the plasmid pAT84 (Flanagan and Rebbitts, *supra*) into the Hind III site of the intermediate vector pSP72 (Promega). This plasmid was then used as the template DNA from which a shortened version of the gamma 4 constant region was obtained using the standard PCR amplification procedures described above and the primer pairs indicated in Figure 4. Eukaryotic expression vectors were constructed as described below such that the heavy chain immunoglobulin molecule was transcribed from a plasmid carrying the neomycin (G418) (Rothstein and Reznikoff, Cell 23: 191-199 [1981]) resistance marker, while the light chain immunoglobulin was transcribed from a plasmid carrying the hygromycin B resistance marker (Gritz and Davies, Gene 25: 179-188 [1983]). With the exception of the drug resistance portion of these plasmids they are identical.

The progenitor of the immunoglobulin expression vectors was the pD5 eukaryotic expression vector (Berkner and Sharp, Nucl. Acids Res. 13: 841-857 [1985]) which contained the origin of adenovirus replication, the SV40 enhancer domain, the adenovirus major late promoter, the adenovirus 2 tripartite leader, a 5' splice donor from the adenovirus third leader and a 3' splice acceptor derived from an immunoglobulin locus, a multiple cloning site, and the SV40 late polyadenylation signal (Figure 10). The origin of replication was removed by digestion with Eco RI and Kpn I and replaced by two fragments representing the neo selectable marker gene (derived from plasmid pCMVIE-AK1-DHFR (Silberklang et al., Modern Approaches to Animal Cell Technology, Ed. Spier et al., Butterworth, U.K., [1987]) as an Eco RI/Bam HI 1.8 Kb fragment) and the Ig heavy chain enhancer (obtained as a PCR amplified fragment using standard procedures described above and human DNA as the template; the oligodeoxynucleotide primer pair is listed in Figure 4) following its diges-

tion with Bgl II and Kpn I. The resultant expression vector was found to lack a small portion of the TK promoter responsible for the transcription of the neomycin gene. This was replaced by insertion into the Eco RI site of a 0.14 kb PCR amplified fragment derived from the CMVIE-AK1-DHFR DNA using the primer pair also listed in Figure 4. The resultant heavy chain expression vector was subsequently modified by removal of the indicated Hind III and Xba I sites. To convert this neomycin selectable vector into one expressing the hygromycin B selectable marker (Figure 11) the neomycin-resistance cassette was removed by digestion first with Eco RI followed by DNA polymerase-directed fill in of the 5' overhang, then subsequent Sal I digestion. The 1.9 kb hygromycin B expression cassette [TK promoter and TK polyadenylation signal flanking the hygromycin B gene obtained from Gritz and Davies, Gene 25: 179-188(1983), as the 1.9 kb Bam HI fragment in plasmid (pLG90)] was removed from the plasmid pAL-2 by Bam HI digestion and subcloned into the Bam HI site of the intermediate vector pSP72 (Promega). The hygromycin B cassette was removed from this vector by digestion with Sma I and Sal I and cloned into the expression vector linearized as described above to create a blunt end and Sal I end DNA fragment.

Expression of the 1B4 "veneered" kappa light chain was accomplished by transferring this cistron from its position within the pSP72 intermediate vector to the hygromycin B selectable eukaryotic expression vector (Figure 7). A 1.5 kb DNA fragment resulting from the endonuclease digestion of v1B4 VK/pSP72 intermediate vector with Spe I and ClaI was purified by agarose gel electro-phoresis and ligated into the expression vector which had previously been linearized, by digestion with the same two restriction enzymes and agarose gel purified.

The 1B4 "veneered" heavy chain eukaryotic expression vector was constructed in one step (Figure 9) from an existing vector previously constructed to express a chimaeric form of the 1B4 heavy chain. The "veneered" heavy chain variable region created by PCR amplification (Figure 8) was digested with Hind III and Bam HI. The agarose gel purified 0.8 kb fragment was ligated into the Hind III and Bam HI sites of the pD5/IgH-Enhancer/Neo/1B4 VH-Short Human C-Gamma 4 expression vector following its endonuclease digestion with these two enzymes and subsequent purification by agarose gel electrophoresis (Figure 9). Transformants containing both variable and constant regions were identified. Plasmid DNAs were grown (Maniatis et al., *supra*) and purified for transfection into recipient mammalian cells (Maniatis et al., *supra*; Birnion and Doly, *supra*).

1999-2000		2000-2001		2001-2002		2002-2003		2003-2004		2004-2005		2005-2006		2006-2007		2007-2008		2008-2009		2009-2010		2010-2011		2011-2012		2012-2013		2013-2014		2014-2015		2015-2016		2016-2017		2017-2018		2018-2019		2019-2020		2020-2021		2021-2022		2022-2023		2023-2024		2024-2025		2025-2026		2026-2027		2027-2028		2028-2029		2029-2030		2030-2031		2031-2032		2032-2033		2033-2034		2034-2035		2035-2036		2036-2037		2037-2038		2038-2039		2039-2040		2040-2041		2041-2042		2042-2043		2043-2044		2044-2045		2045-2046		2046-2047		2047-2048		2048-2049		2049-2050		2050-2051		2051-2052		2052-2053		2053-2054		2054-2055		2055-2056		2056-2057		2057-2058		2058-2059		2059-2060		2060-2061		2061-2062		2062-2063		2063-2064		2064-2065		2065-2066		2066-2067		2067-2068		2068-2069		2069-2070		2070-2071		2071-2072		2072-2073		2073-2074		2074-2075		2075-2076		2076-2077		2077-2078		2078-2079		2079-2080		2080-2081		2081-2082		2082-2083		2083-2084		2084-2085		2085-2086		2086-2087		2087-2088		2088-2089		2089-2090		2090-2091		2091-2092		2092-2093		2093-2094		2094-2095		2095-2096		2096-2097		2097-2098		2098-2099		2099-2100		2100-2101		2101-2102		2102-2103		2103-2104		2104-2105		2105-2106		2106-2107		2107-2108		2108-2109		2109-2110		2110-2111		2111-2112		2112-2113		2113-2114		2114-2115		2115-2116		2116-2117		2117-2118		2118-2119		2119-2120		2120-2121		2121-2122		2122-2123		2123-2124		2124-2125		2125-2126		2126-2127		2127-2128		2128-2129		2129-2130		2130-2131		2131-2132		2132-2133		2133-2134		2134-2135		2135-2136		2136-2137		2137-2138		2138-2139		2139-2140		2140-2141		2141-2142		2142-2143		2143-2144		2144-2145		2145-2146		2146-2147		2147-2148		2148-2149		2149-2150		2150-2151		2151-2152		2152-2153		2153-2154		2154-2155		2155-2156		2156-2157		2157-2158		2158-2159		2159-2160		2160-2161		2161-2162		2162-2163		2163-2164		2164-2165		2165-2166		2166-2167		2167-2168		2168-2169		2169-2170		2170-2171		2171-2172		2172-2173		2173-2174		2174-2175		2175-2176		2176-2177		2177-2178		2178-2179		2179-2180		2180-2181		2181-2182		2182-2183		2183-2184		2184-2185		2185-2186		2186-2187		2187-2188		2188-2189		2189-2190		2190-2191		2191-2192		2192-2193		2193-2194		2194-2195		2195-2196		2196-2197		2197-2198		2198-2199		2199-2200		2200-2201		2201-2202		2202-2203		2203-2204		2204-2205		2205-2206		2206-2207		2207-2208		2208-2209		2209-2210		2210-2211		2211-2212		2212-2213		2213-2214		2214-2215		2215-2216		2216-2217		2217-2218		2218-2219		2219-2220		2220-2221		2221-2222		2222-2223		2223-2224		2224-2225		2225-2226	
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polymorphonuclear leukocytes (PMNs). Purified murine anti-CD18 monoclonal antibody (50 ug; m1B4) is iodinated using chloramine-T (Hunter, W.M. and Greenwood, F.C., Nature 194: 495-496, 1962), and the radiolabeled antibody purified using a Bio-Sil TSK250 (Biorad, Richmond, CA) gel filtration HPLC column (which fractionates proteins in the range of 1-300 x 10³ daltons) equilibrated in 0.1M phosphate buffer, pH 7.0. Effluent radioactivity is monitored with an in-line detector (Beckman Model 170; Beckman, Fullerton, CA) and total protein measured at OD₂₈₀ with a Kratos Spectroflow 757 detector (Kratos, Mawah, N.J.). A single ¹²⁵I-m1B4 peak composed of coincident OD₂₈₀ and radioactivity tracings characteristically elutes 6 minutes, 30 seconds following sample injection. Specific activity of the product is generally about 10 μCi/μg protein, and 97-99% of the counts are precipitable with 10% trichloroacetic acid. The binding of this radiolabeled antibody is assessed on human PMNs purified on a discontinuous Ficoll/Hypaque gradient (English, D. and Anderson, B.R., J. Immunol. Methods 5: 249-255, 1974) and activated with 100 ng/ml phorbol myristate for 20 minutes at 37°C (Lo, et al., J. Exp. Med. 169: 1779-1793, 1989). To determine the avidity of antibodies for CD18 molecules on the PMN surface, about 1 x 10⁵ activated PMNs are incubated in a buffer such as Hanks balanced salt solution containing 20 mM Hepes (pH 7.2), 0.14 units aprotinin (Sigma Chemical Co.) and 2% human serum albumin (binding buffer) containing 1.3 ng ¹²⁵I-m1B4 (2.8 x 10⁻¹¹M) in the presence of increasing concentrations of unlabeled m1B4 antibody (10⁻⁷ to 10⁻¹⁵M) in a 300 ul reaction volume for about 1 h at about 4°C with constant agitation. Cell bound 1B4 is separated from the unbound antibody by centrifugation through a 0.5M sucrose cushion (4,800 x g, 3 minutes); the tubes are frozen on dry ice, and the tips cut off and counted with an LKB gamma counter. The IC₅₀ of the anti-CD18 antibody for the inhibition of ¹²⁵I-m1B4 antibody binding is calculated using a four parameter fitter program (Rodbard, D. Munson, P.J., and DeLean, In, "Radioimmunoassay and Related Procedures in Medicine", International Atomic Energy Agency, Vienna, vol 1,469-504, 1978). The affinity of the "veneered" anti-CD18 antibody for the CD18 ligand is determined in a similar manner using murine ¹²⁵I-m1B4 antibody and increasing quantities, as determined by the trapping Elisa, of unlabeled "veneered" anti-CD18 antibody. The results of the binding assays are shown in Figure 13 and indicate that the avidity of the "veneered" heavy chain and light chain recombinant 1B4 antibody is equivalent to that of the murine 1B4 monoclonal antibody.

The "veneered" heavy and light chain expression vectors were co-transfected into CV1P mon-

key kidney cells using 20 µg of each plasmid to prepare 2 mL of the calcium phosphate precipitated solution. One mL was placed in the medium overlaying each 100 mm dish of CV1P cells. After 4 hr at 37°C the medium was replaced with 1 mL of 15% glycerol in 1 x HBS (Hepes buffered salt). Following the 3 min glycerol shock, 10 mL of PBS as added, the cell monolayers were aspirated, washed once with 10 mL of PBS, and re-fed with fresh medium (DMEM + 10% heat inactivated new born calf serum) containing 200 µg of hygromycin B and 800 µg of G418 per mL. Cloning cylinders (Fishney, In, Culture of Animal Cells, Alan R. Liss, Inc. New York, 1983) were used to isolate individual colonies prior to their expansion and subsequent assay for productivity. Two clones, #11 and #48, were found to express sufficient amounts of v1B4 to warrant their expansion and ultimate accessioning.

Claims

1. A method for identifying differences in mammalian species specific surface amino acid residues on an immunoglobulin comprising:
 - a. comparing the framework amino acids of a variable domain of a first mammalian species with the variable domains of a second mammalian species;
 - b. determining the subgroups of the second mammalian species to which the first mammalian species most closely corresponds;
 - c. determining the second mammalian species sequence which is most similar to the first mammalian species sequence;
 - d. identifying amino acid residues of the first mammalian species which differ from the amino acid residues of the second mammalian species, with said amino acids being mostly exposed or completely exposed on the immunoglobulin surface;
 - e. identifying only those amino acid residues which are not within a complementarity-determining region or are not directly adjacent to a complementarity-determining region.
2. The method of claim 1 wherein the first mammalian species is mouse.
3. The method of claim 1 wherein the second mammalian species is human.
4. A method for converting an immunoglobulin having the immunogenicity of a first mammalian species to an antibody having the immunogenicity of a second mammalian species comprising:
 - a. replacing the amino acid residues in a first mammalian species framework which differ from the amino acid residues of a second mammalian species with the corresponding amino acid residues from the most similar second mammalian species as identified by the method of claim 1.
5. The method of claim 2 wherein the first mammalian species is mouse.
6. The method of claim 2 wherein the second mammalian species is human.
7. A method comprising:
 - a. preparing a DNA sequence encoding a veneered immunoglobulin having specificity for a known antigen wherein the surface amino acid residues of a first mammalian species which differ from the surface amino acid residues of a second mammalian species are replaced with the corresponding amino acids residues from the most similar second second mammalian species sequence as identified by the method of claim 1;
 - b. inserting the sequence into a replicable expression vector operably linked to a suitable promoter compatible with a host cell;
 - c. transforming the host cell with the vector of b;
 - d. culturing the host cell; and
 - e. recovering the veneered immunoglobulin from the host cell culture.
8. The method of claim 7 wherein the first mammalian species is mouse.
9. The method of claim 7 wherein the second mammalian species is human.
10. A composition comprising a veneered immunoglobulin having a specificity for a known antigen.
11. A DNA sequence encoding veneered 1B4 antibody.
12. A veneered murine 1B4 antibody exhibiting the antigenicity of human antibody of fragments thereof.

Fig. 1a

Position	Fractional Accessibility				Residues In Subgroup		
	KOL		J539		I	II	III
	Residue Exposure		Residue Exposure				
1	E	1.00 Ex	E	1.00 Ex	Q	Q	E
2	V	0.23 mB	V	0.37 mB	V	V	V M
3	Q	0.82 Ex	K	0.82 Ex	Q	T Q	Q
4	L	0.00 Bu	L	0.10 Bu	L	L	L
5	V	0.87 Ex	L	1.00 Ex	V	RQKT	VL
6	Q	0.00 Bu	E	0.09 Bu	Q	E	E
7	S	0.94 Ex	S	0.94 Ex	S	S	S
8	G	1.00 Ex	G	1.00 Ex	G	G	G
9	G	0.00 Bu	G	0.00 Bu	A	P	G
10	G	1.00 Ex	G	1.00 Ex	E	AGT	GA
11	V	0.90 Ex	L	0.81 Ex	V	L	LF
12	V	0.25 mB	V	0.25 mB	K	V	V
13	Q	0.71 mE	Q	0.87 Ex	K	K	Q
14	P	0.59 PB	P	0.64 mE	P	P	P
15	G	1.00 Ex	G	1.00 Ex	G	TS	G
16	R	0.73 mE	G	1.00 Ex	SA	EQ	G
17	S	0.66 mE	S	0.75 mE	S	T	S
18	L	0.28 mB	L	0.26 mB	V	L	L
19	R	0.66 mE	K	0.75 mE	RK	TS	RK
20	L	0.00 Bu	C	0.00 Bu	V	L	L
21	S	0.71 mE	S	0.82 Ex	S	T	S
22	C	0.00 Bu	C	0.00 Bu	C	C	C
23	S	1.00 Ex	A	1.00 Ex	K	T	A
24	S	0.00 Bu	A	0.00 Bu	ATV	FV	A
25	S	0.87 Ex	S	1.00 Ex	S	S	S
26	G	1.00 Ex	G	1.00 Ex	G	G	G
27	F	0.10 Bu	F	0.10 Bu	GYD	FLG	F
28	I	0.85 Ex	D	0.72 mE	T	S	TN
29	F	0.00 Bu	F	0.00 Bu	F	LI	F
30	S	0.74 mE	S	0.83 Ex	SNVI	S	S
36	W	0.00 Bu	W	0.00 Bu	W	W	W
37	V	0.00 Bu	V	0.00 Bu	V	I	V
38	R	0.10 Bu	R	0.31 mB	R	R	R
39	Q	0.15 Bu	Q	0.28 mB	Q	Q	Q
40	A	0.95 Ex	A	0.75 mE	A	P	A
41	P	0.90 Ex	P	0.73 mE	P	P	PS
42	G	1.00 Ex	G	1.00 Ex	G	G	G
43	K	0.86 Ex	K	0.86 Ex	QRKH	KR	K
44	G	1.00 Ex	G	1.00 Ex	G	AG	GS
45	L	0.00 Bu	L	0.00 Bu	L	L	L

009920-2290T940

Fig. 1b

Fractional Accessibility					Residues In Subgroup					
Position	KOL		J539		I		II		III	
	Residue	Exposure	Residue	Exposure						
46	E	0.75 mE	E	0.73 mE	E		E		E	
47	W	0.10 Bu	W	0.04 Bu	W		W		W	
48	V	0.00 Bu	I	0.00 Bu	MV		LI		V	
49	A	0.00 Bu	G	0.00 Bu	G		AG		G S A	
66	R	0.36 mB	K	0.51 pB	R		R		R	
67	F	0.00 Bu	F	0.00 Bu	V		LV		F	
68	T	0.87 Ex	I	0.88 Ex	T		T		T	
69	I	0.00 Bu	I	0.00 Bu	VMI		IV		I	
70	S	0.78 mE	S	0.79 mE	TS		ST		S	
71	R	0.11 Bu	R	0.00 Bu	RLA		KV		R	
72	N	0.61 mE	D	0.55 pB	DK		D		D N	
73	D	0.44 pB	N	0.43 pB	PETAS		T		D N	
74	S	0.85 Ex	A	0.97 Ex	S		S		S	
75	K	0.88 Ex	K	0.77 mE	TF		KR		K	
76	N	0.69 mE	N	0.68 mE	NST		N		N	
77	T	0.41 pB	S	0.33 mB	TQ		Q		T	
78	L	0.00 Bu	L	0.00 Bu	AV		VF		L A	
79	F	0.45 pB	Y	0.35 mB	Y		VS		Y F	
80	L	0.00 Bu	L	0.00 Bu	M		L		L	
81	Q	0.53 pB	Q	0.69 mE	E		TKSIN		Q	
82	M	0.00 Bu	M	0.00 Bu	L		ML		M	
82a	D	0.73 mE	S	0.58 pB	SVRT		TSNIR		D N D	
82b	S	0.98 Ex	K	0.96 Ex	S		NS		S	
82c	L	0.00 Bu	V	0.00 Bu	L		VM		L	
83	R	0.73 mE	R	0.83 Ex	RFI		DT		R E	
84	P	0.75 mE	S	0.90 Ex	S		PA		P A	
85	E	0.82 Ex	E	0.90 Ex	E		VA		E D	
86	D	0.00 Bu	D	0.11 Bu	D		D		D	
87	T	0.54 pB	T	0.47 pB	T		T		T	
88	G	1.00 Ex	A	0.00 Bu	A		A		A	
89	V	0.58 PB	L	0.63 mE	V		TV		V L	
90	Y	0.00 Bu	Y	0.00 Bu	Y		Y		Y	
91	F	0.00 Bu	Y	0.08 Bu	Y		Y		Y	
92	C	0.00 Bu	C	0.00 Bu	C		C		C	
93	A	0.00 Bu	A	0.00 Bu	A		A		A T	
94	R	0.17 Bu	R	0.15 Bu	R		RH		R P	
					JH1	JH2	JH3	JH4	JH5	JH6
103	W	0.09 Bu	W	0.07 Bu	W	W	W	W	W	W
104	G	0.00 Bu	G	1.00 Ex	G	G	G	G	G	G

Fig. 1c

Position	Fractional Accessibility				Residues In Subgroup					
	Residue	KOL Exposure	Residue	J539 Exposure	I	II		III		
					JH1	JH2	JH3	JH4	JH5	JH6
105	Q	0.93 Ex	Q	0.99 Ex	Q	R	Q	Q	Q	Q
106	G	0.00 Bu	G	0.00 Bu	G	G	G	G	G	G
107	T	0.22 mB	T	0.26 mB	T	T	T	T	T	T
108	P	0.99 Ex	L	0.67 mE	L	L	M	L	L	T
109	V	0.00 Bu	V	0.00 Bu	V	V	V	V	V	V
110	T	0.76 mE	T	0.69 mE	T	T	T	T	T	T
111	V	0.00 Bu	V	0.00 Bu	V	V	V	V	V	V
112	S	0.98 Ex	S	0.74 mE	S	S	S	S	S	S
113	S	0.94 Ex	A	0.84 Ex	S	S	S	S	S	S

005020-8520460

Fig. 2a

Position	Residue Exposure		Residues In Subgroup					
			I	II	III	IV	V	VI
1	Q	1.00 Ex	Q	Q	SF	-	Q	ND
2	S	1.00 Ex	S	S	Y	S	S	F
3	V	0.77 mE	V	A	E	E	A	M
4	L	0.00 Bu	L	L	L	L	L	L
5	T	0.92 Ex	T	T	TK	T	T	T
6	Q	0.00 Bu	Q	Q	Q	Q	Q	Q
7	P	0.62 mE	P	P	P	D	P	P
8	P	1.00 Ex	P	ARP	P	P	P	H
9	S	1.00 Ex	S	S	S	A	S	S
10	-	-	-	-	-	-	-	-
11	A	0.34 mB	AV	V	V	V	A	V
12	S	0.71 mE	S	S	S	S	S	S
13	G	1.00 Ex	GA	G	VL	V	G	E
14	T	0.73 mE	TA	S	SA	A	S	S
15	P	0.75 mE	P	P	PA	L	PL	P
16	G	1.00 Ex	G	G	G	G	G	G
17	Q	0.69 mE	Q	Q	Q	Q	Q	K
18	R	0.79 mE	R	S	T	T	S	T
19	V	0.21 mB	V	IV	A	V	V	V
20	T	0.62 ME	T	T	RM	R	T	T
21	I	0.00 Bu	I	I	I	I	I	IFM
22	S	0.92 Ex	S	S	T	T	S	S
23	C	0.00 Bu	C	C	C	C	C	C
35	W	0.00 Bu	W	W	W	W	W	W
36	Y	0.00 Bu	Y	YF	Y	Y	Y	Y
37	Q	0.46 pB	Q	Q	Q	Q	Q	Q
38	Q	0.00 Bu	QH	Q	QE	Q	Q	Q
39	L	0.75 mE	LV	H	KR	K	H	R
40	P	0.91 Ex	P	P	PS	P	PA	P
41	G	1.00 EX	G	G	G	G	G	G
42	M	0.74 mE	T	K	QR	Q	RK	SRG
43	A	0.62 mE	A	A	A	A	A	A
44	P	0.00 Bu	P	P	P	P	P	P
45	K	0.95 Ex	K	K	V	L	K	T
46	L	0.23 mB	L	L	MLP	L	LV	T
47	L	0.15 Bu	L	MIL	V	V	VI	V
48	I	0.00 Bu	I	I	IV	I	I	I
49	Y	0.39 mB	Y	YF	Y	Y	FY	Y
57	G	1.00 Ex	G	G	GE	G	G	G
58	V	0.14 Bu	VI	VI	IV	I	V	V
59	P	0.70 mE	P	SP	P	P	P	P
60	D	0.95 Ex	D	DNL	EOA	D	D	D
61	R	0.31 mB	R	R	R	R	R	R

0049020-22201950

Position			Residues In Subgroup					
	Residue	Exposure	I	II	III	IV	V	VI
62	F	0.12 Bu	F	F	F	F	F	F
63	S	0.85 Ex	S	S	S	S	S	S
64	G	0.00 Bu	GA	G	GS	G	G	G
65	S	1.00 Ex	S	S	SY	S	S	S
66	K	0.41 pB	K	K	TSN	S	K	IF*
67	S	1.00 Ex	S	S	S	S	S	S
68	G	1.00 Ex	G	G	G	G	DG	S
69	A	0.71 mE	T	N	TN	H	N	S
70	S	1.00 Ex	S	T	TKS	T	T	S
71	A	0.00 Bu	A	A	AV	A	A	A
72	S	1.00 Ex	ST	S	TI	S	S	A
73	L	0.00 Bu	L	L	L	L	L	L
74	A	0.74 mE	A	T	T	T	T	L
75	I	0.00 Bu	I	I	I	I	V	I
76	G	1.00 Ex	ST	S	SN	T	S	S
77	G	1.00 Ex	G	G	GR	G	G	G
78	L	0.00 Bu	L	L	VA	A	L	L
79	Q	0.76 mE	QR	Q	QE	Q	RQ	KQT
80	S	1.00 Ex	ST	A	AV	A	A	T
81	E	0.78 mE	EG	E	EG	E	E	E
82	D	0.09 Bu	D	D	D	D	D	D
83	E	0.64 mE	E	E	E	E	E	E
84	T	0.34 mB	A	A	A	A	A	A
85	D	0.30 mB	D	D	D	D	D	D
86	Y	0.00 Bu	Y	Y	Y	Y	Y	Y
87	Y	0.16 Bu	Y	Y	YF	Y	Y	Y
88	C	0.00 Bu	C	C	C	C	C	C
			JL-1	JL-2	JL-3	JL-4	JL-5	
98	F	0.04 Bu	F	F	F	F	F	
99	G	0.00 Bu	G	G	G	G	G	
100	T	0.59 pB	T	G	G	S	S	
101	G	1.00 Ex	G	G	G	G	G	
102	T	0.00 Bu	T	T	T	T	T	
103	K	0.82 Ex	K	K	K	Q	Q	
104	V	0.00 Bu	V	L	L	L	L	
105	T	0.86 Ex	T	T	T	T	T	
106	V	0.19 Bu	V	V	V	V	V	
106a	L	0.70 mE	L	L	L	L	L	
107	G	1.00 Ex	G	G	G	S	G	

66a D
66b S R D

Position	Residues In Subgroup					
	Residue	Exposure	I	II	III	IV
1	E	0.99 Ex	D	D	E	D
2	I	0.16 Bu	I	I	I	I
3	V	0.87 Ex	Q	V	V	V
4	L	0.00 Bu	M	M	L	M
5	T	0.80 mE	T	T	T	T
6	Q	0.00 Bu	Q	Q	Q	Q
7	S	0.89 Ex	S	S	S	S
8	P	0.67 mE	P	P	P	P
9	A	1.00 Ex	S	L	G	D ^N
10	I	0.94 Ex	S	L	T	S
11	T	0.30 mB	L	L	L	L
12	A	0.59 pB	S	P	L	A
13	A	0.00 Bu	A	V	L	V
14	S	0.78 mE	S	T	S	S
15	L	0.79 mE	V	P	P	L
16	G	1.00 Ex	G	G	G	G
17	Q	0.64 mE	D	E	E	E
18	K	0.74 mE	R	P	R	R
19	V	0.22 mB	V	A	A	A
20	T	0.65 mE	T	S	T	T
21	I	0.00 Bu	I	I	L	I
22	T	0.69 mE	T	S	S	N
23	C	0.00 Bu	C	C	C	C
35	W	0.00 Bu	W	W	W	W
36	Y	0.00 Bu	Y	Y	Y	Y
37	Q	0.14 Bu	Q	L	Q	Q
38	Q	0.24 mB	Q	Q	Q	Q
39	K	0.69 mE	K	K	K	K
40	S	1.00 Ex	P	P	P	P
41	G	1.00 Ex	G	G	G	G
42	T	0.90 Ex	K	Q	Q	Q
43	S	0.30 mB	A	S	A	P
44	P	0.00 Bu	P	P	P	P
45	K	0.90 Ex	K	Q ^{ER}	R	K
46	P	0.43 pB	L	L	L	L
47	W	0.16 Bu	L	L	L	L
48	I	0.00 Bu	I	I	I	I
49	Y	0.42 pB	Y	Y	Y	Y
57	G	1.00 Ex	G	G	G	G
58	V	0.13 Bu	V	V	I	V
59	P	0.61 mE	P	P	P	P
60	A	1.00 Ex	S	D	D	D
61	R	0.36 mB	R	R	R	R
62	F	0.00 Bu	F	F	F	F
63	S	0.94 Ex	S	S	S	S

Position	Residues In Subgroup					
	Residue	Exposure	I	II	III	IV
1	E	0.99 Ex	D	D	E	D
2	I	0.16 Bu	I	I	I	I
3	V	0.87 Ex	Q	V	V	V
4	L	0.00 Bu	M	M	L	M
5	T	0.80 mE	T	T	T	T
6	Q	0.00 Bu	Q	Q	Q	Q
7	S	0.89 Ex	S	S	S	S
8	P	0.67 mE	P	P	P	P
9	A	1.00 Ex	S	L	G	D ^N
10	I	0.94 Ex	S	L	T	S
11	T	0.30 mB	L	L	L	L
12	A	0.59 pB	S	P	L	A
13	A	0.00 Bu	A	V	L	V
14	S	0.78 mE	S	T	S	S
15	L	0.79 mE	V	P	P	L
16	G	1.00 Ex	G	G	G	G
17	Q	0.64 mE	D	E	E	E
18	K	0.74 mE	R	P	R	R
19	V	0.22 mB	V	A	A	A
20	T	0.65 mE	T	S	T	T
21	I	0.00 Bu	I	I	L	I
22	T	0.69 mE	T	S	S	N
23	C	0.00 Bu	C	C	C	C
35	W	0.00 Bu	W	W	W	W
36	Y	0.00 Bu	Y	Y	Y	Y
37	Q	0.14 Bu	Q	L	Q	Q
38	Q	0.24 mB	Q	Q	Q	Q
39	K	0.69 mE	K	K	K	K
40	S	1.00 Ex	P	P	P	P
41	G	1.00 Ex	G	G	G	G
42	T	0.90 Ex	K	Q	Q	Q
43	S	0.30 mB	A	S	A	P
44	P	0.00 Bu	P	P	P	P
45	K	0.90 Ex	K	Q ^{ER}	R	K
46	P	0.43 pB	L	L	L	L
47	W	0.16 Bu	L	L	L	L
48	I	0.00 Bu	I	I	I	I
49	Y	0.42 pB	Y	Y	Y	Y
57	G	1.00 Ex	G	G	G	G
58	V	0.13 Bu	V	V	I	V
59	P	0.61 mE	P	P	P	P
60	A	1.00 Ex	S	D	D	D
61	R	0.36 mB	R	R	R	R
62	F	0.00 Bu	F	F	F	F
63	S	0.94 Ex	S	S	S	S

Position	Residue Exposure		Residues In Subgroup				
			I	II	III	IV	
64	G	0.00 Bu	G	G	G	G	
65	S	1.00 Ex	S	S	S	S	
66	G	1.00 Ex	G	G	G	G	
67	S	1.00 Ex	S	S	S	S	
68	G	1.00 Ex	G	G	G	G	
69	T	0.75 mE	T	T	T	T	
70	S	0.98 Ex	DEQ	D	D	D	
71	Y	0.09 Bu	F	F	F	F	
72	S	0.70 mE	T	T	T	T	
73	L	0.00 Bu	L	L	L	L	
74	T	0.43 pB	T	K	T	T	
75	I	0.00 Bu	I	I	I	I	
76	N	0.83 Ex	S	S	S	S	
77	T	0.83 Ex	S	R	R	S	
78	M	0.00 Bu	L	V	L	L	
79	E	0.63 mE	Q	EQ	E	Q	
80	A	0.96 Ex	P	A	P	A	
81	E	0.91 Ex	ED	E	E	E	
82	D	0.13 Bu	D	D	D	D	
83	A	0.55 pB	FI	V	F	V	
84	A	0.00 Bu	A	G	A	A	
85	I	0.58 pB	T	V	V	V	
86	Y	0.00 Bu	Y	Y	Y	Y	
87	Y	0.11 Bu	Y	Y	Y	Y	
88	C	0.00 Bu	C	C	C	C	
			JK-1	JK-2	JK-3	JL-4	JL-5
98	F	0.00 Bu	F	F	F	F	F
99	G	1.00 Ex	G	G	G	G	G
100	A	1.00 Ex	Q	Q	P	G	Q
101	G	0.00 Bu	G	G	G	G	G
102	T	0.00 Bu	T	T	T	T	T
103	K	0.79 mE	K	K	K	K	R
104	L	0.00 Bu	V	L	V	V	L
105	E	0.89 Ex	E	E	D	E	E
106	L	0.44 pB	I	I	I	I	I
106a	-	-	-	-	-	-	-
107	K	0.77 mE	K	K	K	K	K

Mouse Light Chain Variable Region

5'-TCT CGGATCCGA (CT)AT (TC)GT G(AC)T (GC)AC CCA (GA) -3'
Bam H1

5- TCT CAA GCT TTG GTG GCA AGA T(GA)G ATA CAG TTG GTG CAG C-3'
Hind III

5' upstream primer - FR1 of variable region

1) 5'- TTC TGG ATC C(CG)A GGT (GCT)CA (AG)CT G(AC)A G(GC)A GTC (TA)GG -3'
Bam H1

ii) 5'- TTC TGG ATC C(CG)A GGT (GCT)AA GCT GGT G(GC)A GTC (TA)GG -3'
Bam H1

5'- TCT CAA GCT TAC CGA TGG (GA)GC TGT TGT TTT GGC -3'
Hind III

SHORTEN VERSION OF THE IgG4 HEAVY CHAIN CONSTANT REGION

5'- ATT TGG ATC C TC TAG A CATCG CGG ATA GAC AAG AAC -3'
Bam H1 Xba I

5'-AAT AAT GCG GCG GC A TCG AT G AGC TCA AGT ATG TAG ACG GGG TAC G-3'

Not I Cla I Sac I

5'-TAT AGAATTC GGTAC CCT TCA TCC CCG TGG CCC G-3'

5'-TGC GTG TTC GAA TTC GCC-3'
Eco RI

5'-TTT TAG ATC T GT CGA CAG ATG GCC GAT CAG AAC CAG-3'
Bgl II Sal I

5'-TTG GTC GAC GGT ACC AAT ACA TTT TAG AAG TCG AT -3'

Sal I Kpn I

5'-TCT CGG ATC CTC TAG AAG AAT GGC TGC AAA GAG C-3'
5'-TCT CGC TAG CGG ATC CTT GCA GAG GAT GAT AGG G-3'

Fig. 5

Oligodeoxynucleotides for PCR Amplification of the LEN Light Chain
Variable Region

S1 5'-CAT TCG CTT ACC AGA TCT AAG CTT ACT AGT GAG ATC ACA GTT CTC TCT AC-3'
V9 5'-TGG CTC TGC AGC TGA TGG TG-3'

V10 5'-CAC CAT CAG CTG CAG AGC CA-3'
V11 5'-CTG TCT GGG ATC CCA GAT TC-3'

V12 5'-GAA TCT GGG ATC CCA GAC AG-3'
V13 5'-GTT GCA ACA TCT TCA GCC TCC ACG CTG CTG ATG-3'

V14 5'-GTG GAG GCT GAA GAT GTT GCA ACT TAT TAC TG-3'
I3 5'-GAA TGT GCC TAC TTT CTA GAG GAT CCA ACT GAG GAA GCA AAG-3'

A1 5'-CAT TCG CTT ACC AGA TCT-3'
A2 5'-GAA TGT GCC TAC TTT CTA G-3'

Oligodeoxynucleotides for PCR Amplification of the m1B4
Heavy Chain Variable Region

V1 5'-CCC TCC AGG CTT CAC TAA GTC TCC CCC-3'

V2 5'-TTA GTG AAG CCT GGA GGG TCC CTG AAA CTC-3'
V3 5'-GCC CCT TCC CAG GAG CTT GCC GAA CCC AAG ACA TG-3'

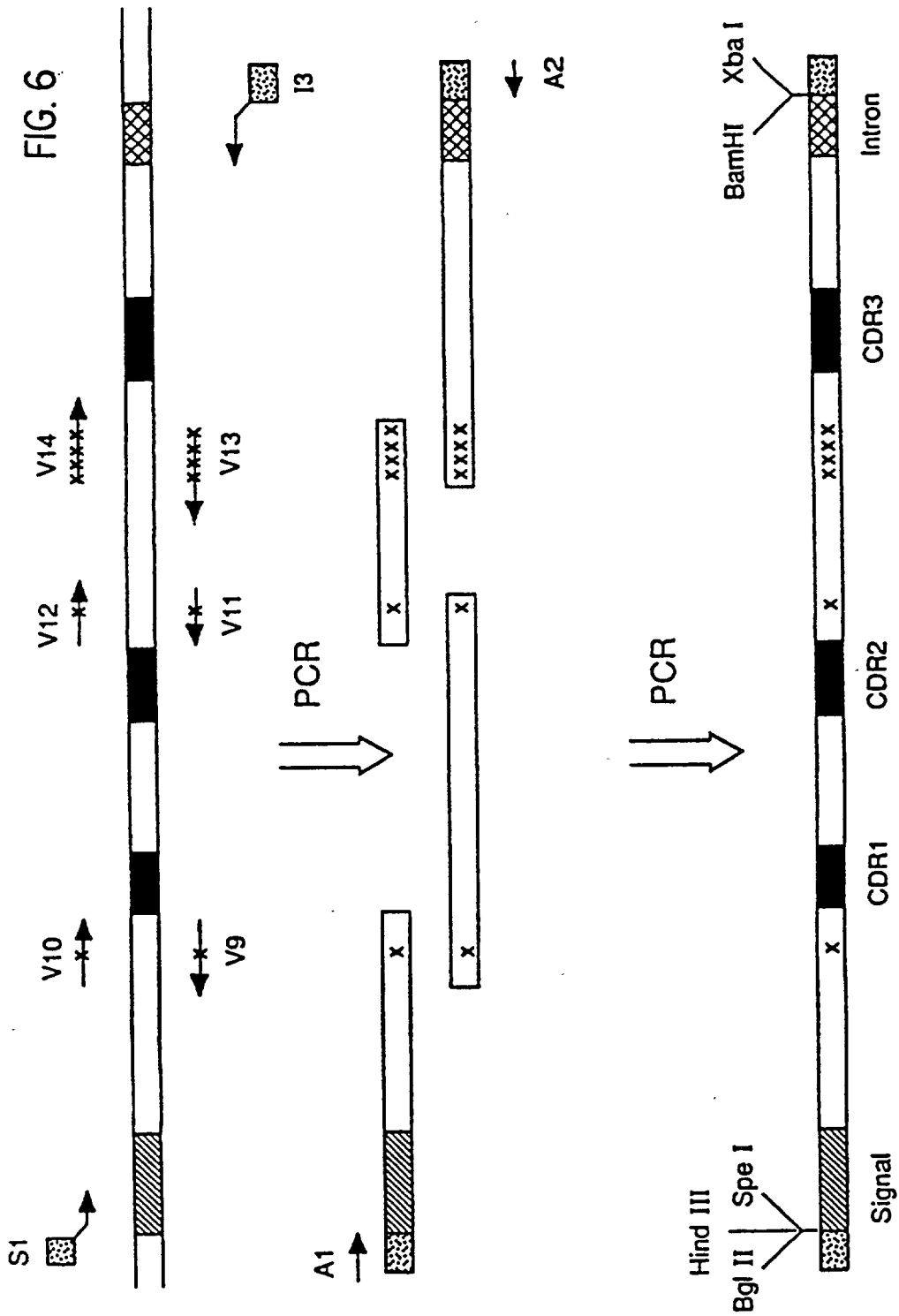
V4 5'-AAG CTC CTG GGA AGG GGC TGG AGT TGG TCG CAG CC-3'
V5 5'-TGT TCA TTT GTA GGT ACA GGG TGT TCT TGG AAT TGT CTC TGG AGA TGG TG-3'

V6 5'-TGT ACC TAC AAA TGA ACA GTC TGA GGG CTG AGG ACA CAG CCT TGT ATT-3'
V7 5'-CTG TGA GAA GGG TGC CTT GGC CCC AGT AG-3'

V8 5'-AAG GCA CCC TTC TCA CAG TCT CCT CAG GTG-3'
I2 5'-GAA TGT GCC TAC TTA AGC TTT CTA GAG GAT CCT ATA AAT CTC TGG CCA TG-3'

S1, A1, and A2, as above

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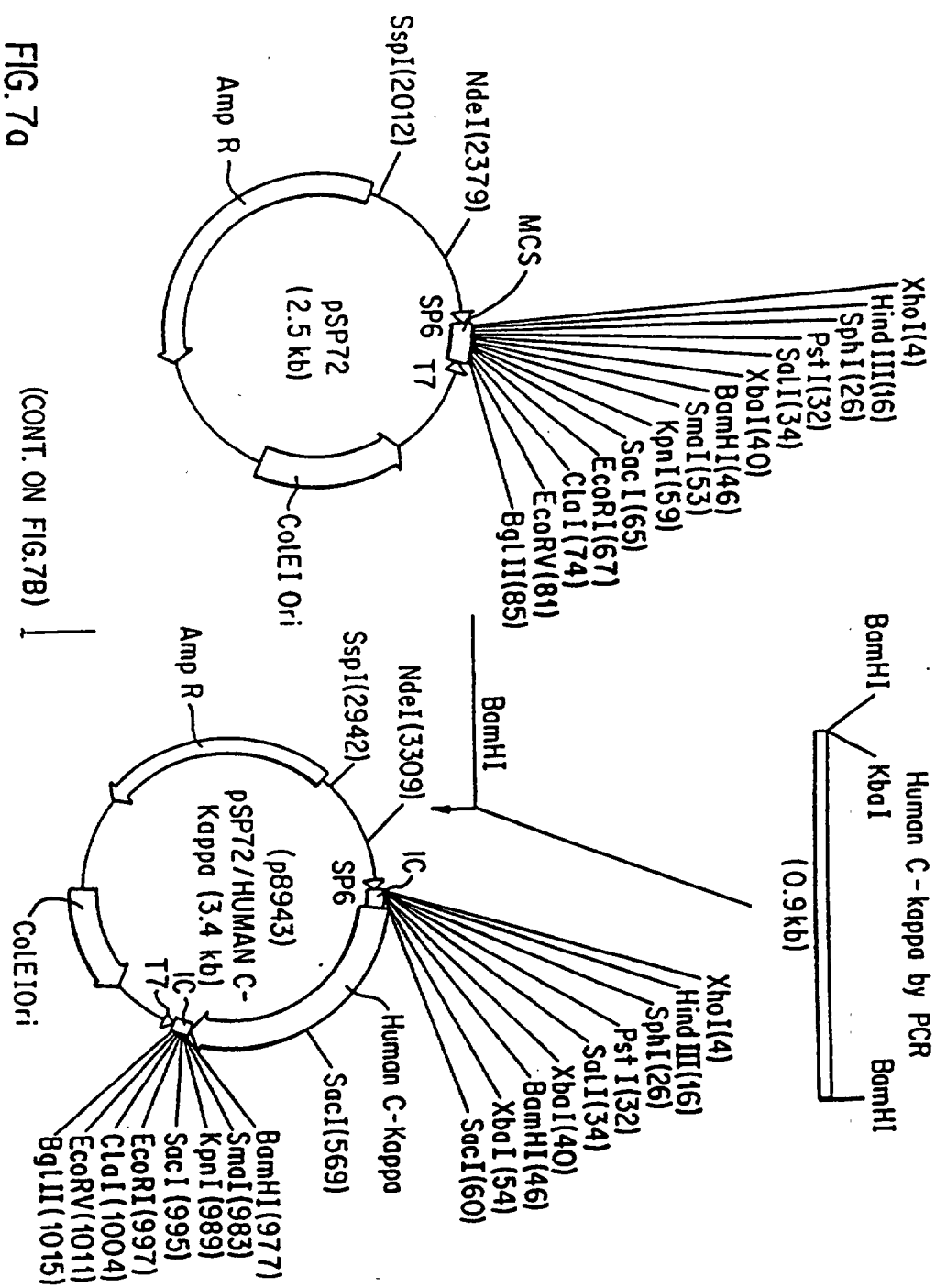
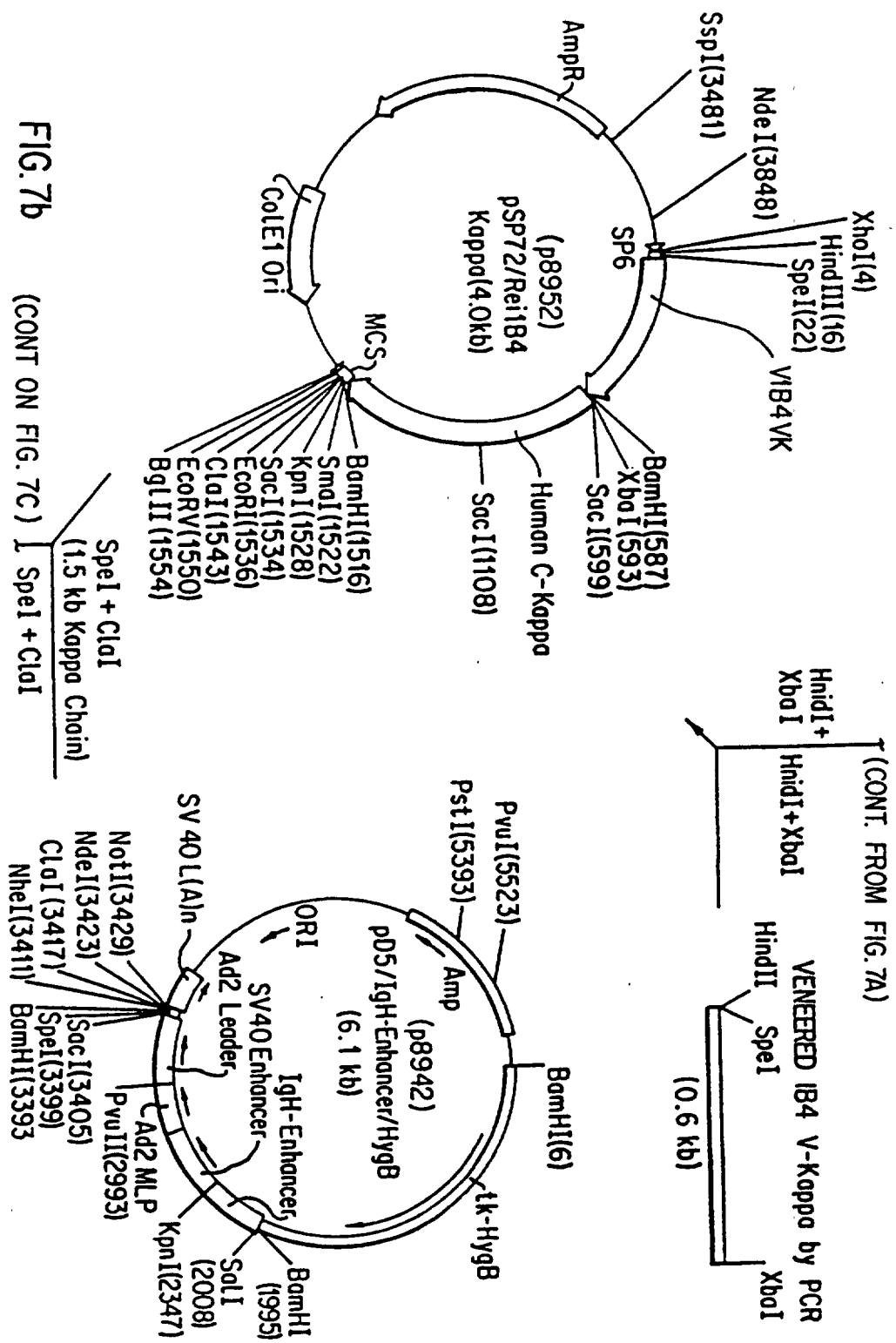


FIG. 7a

(CONT. ON FIG.7B)



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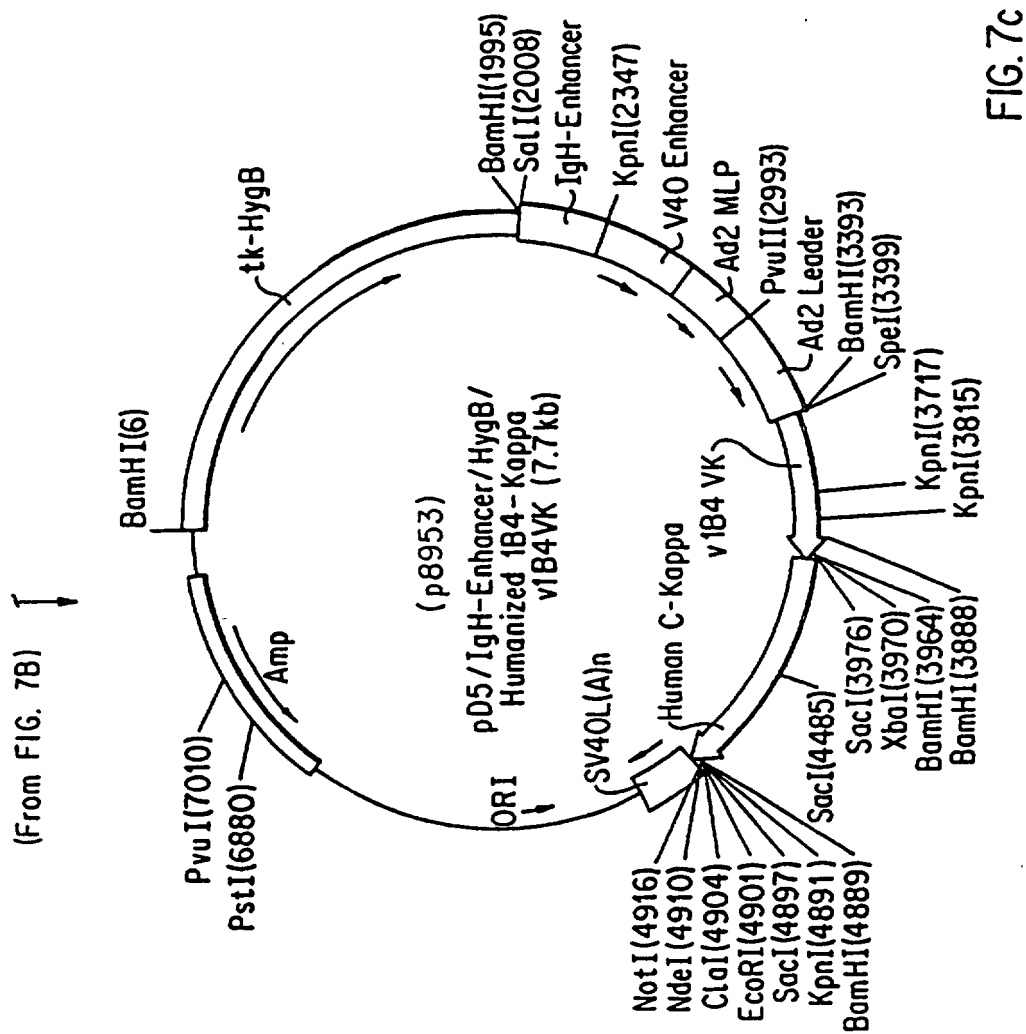
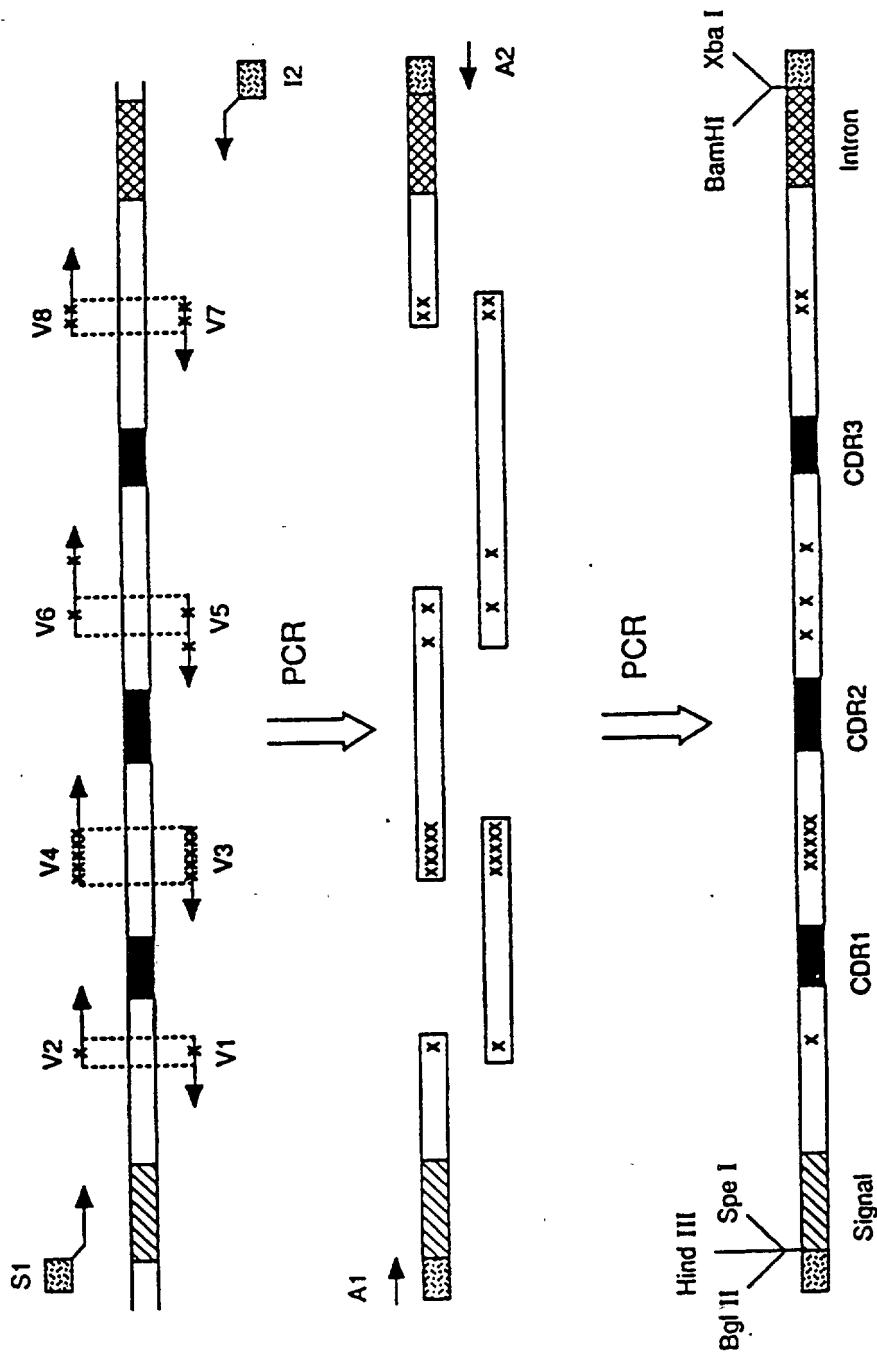


FIG. 7c

Fig. 8

VENEER 1B4 VH



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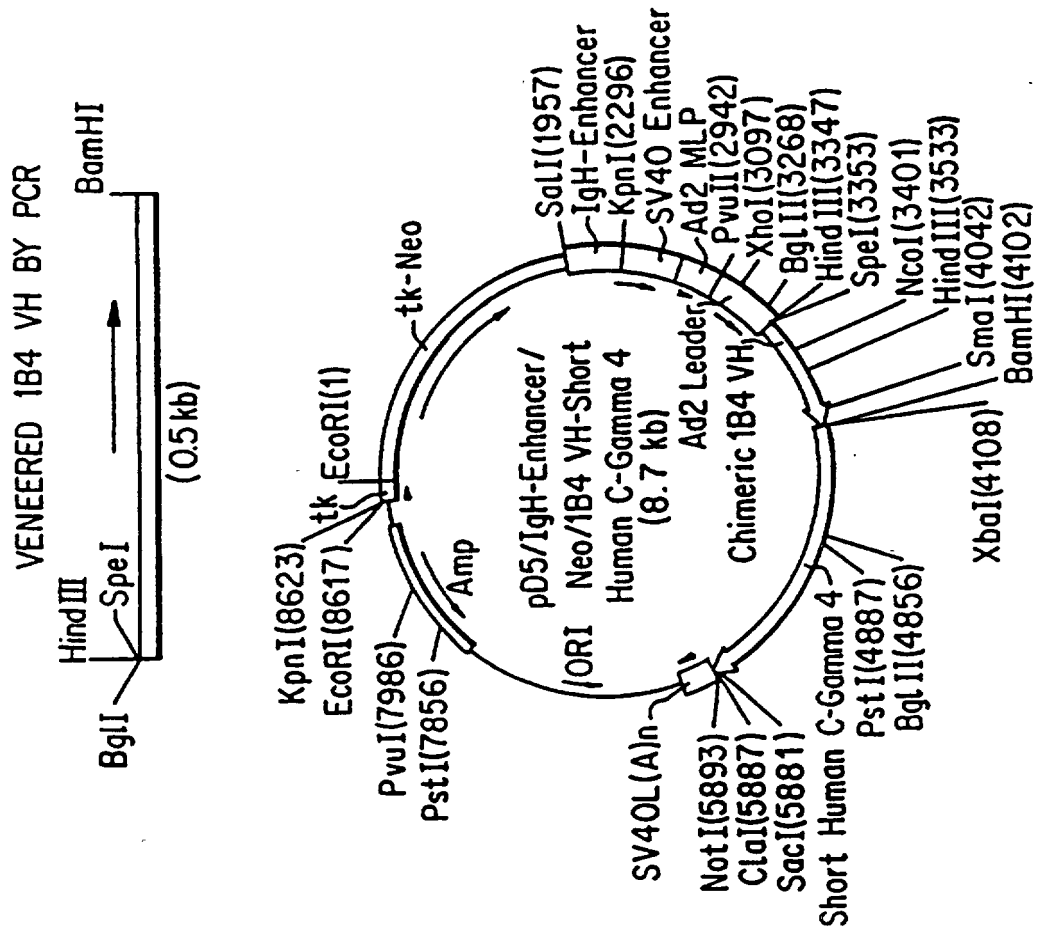


FIG. 9a

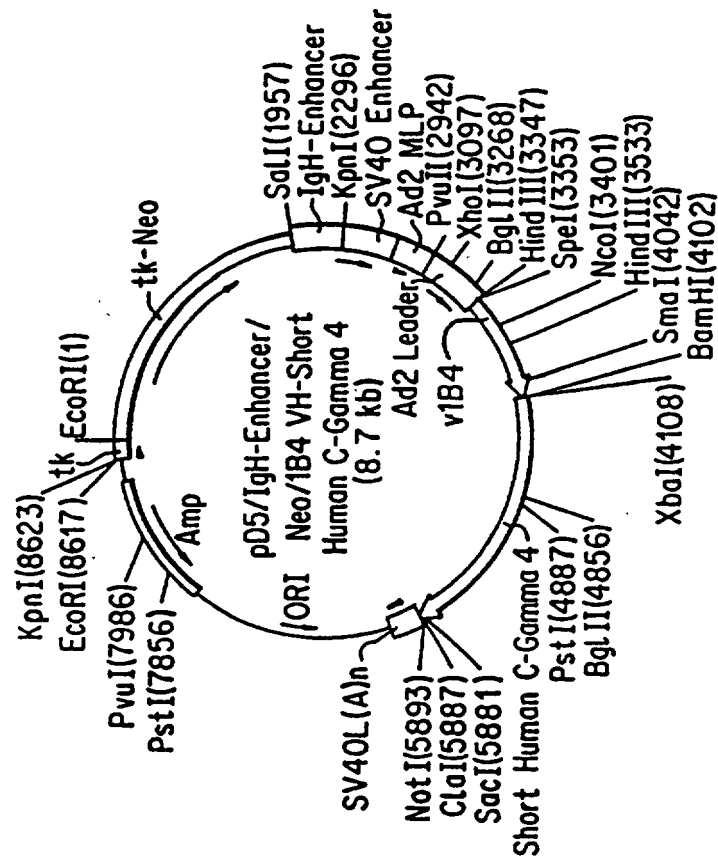


FIG. 9b

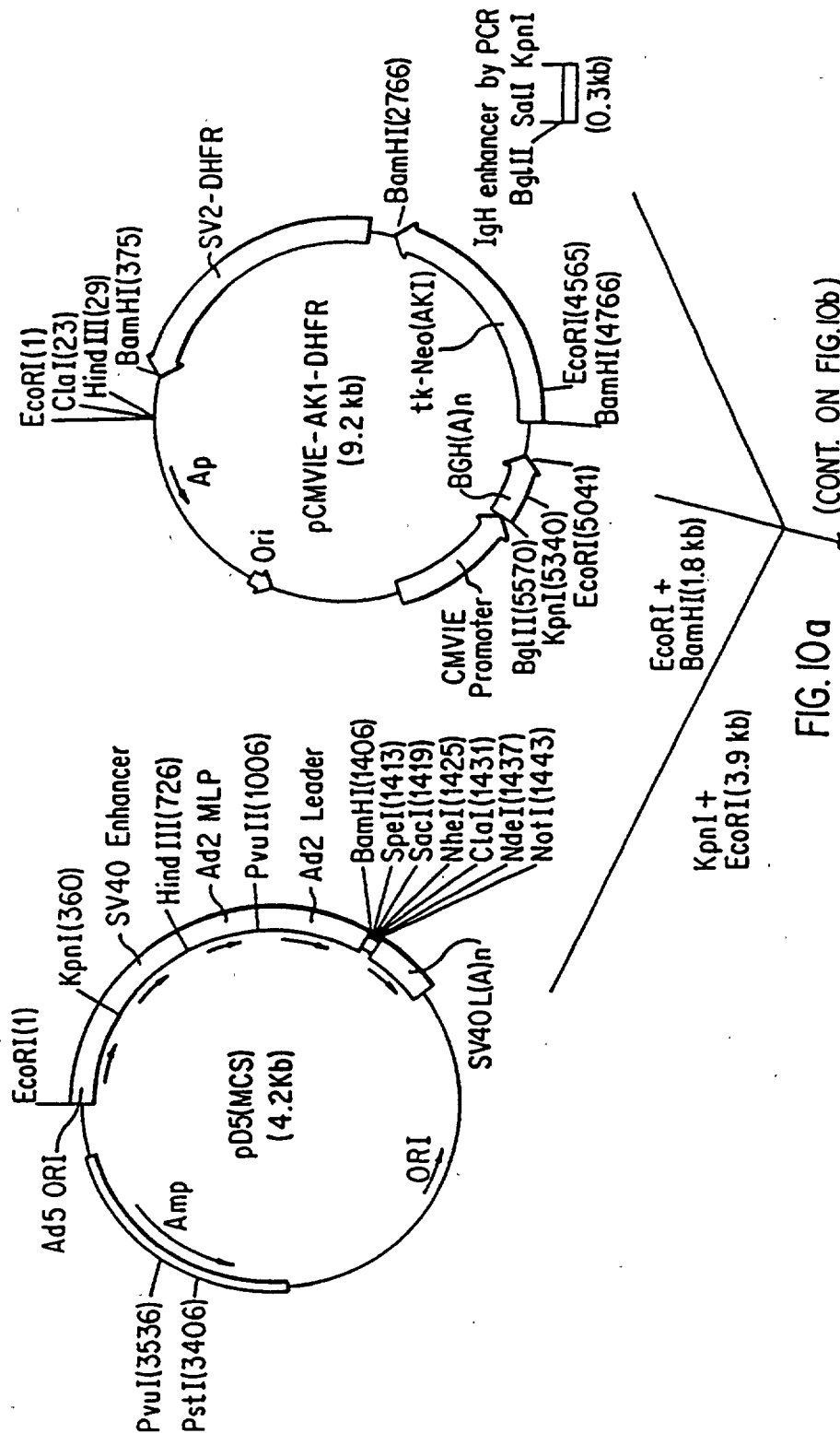


FIG. 10a (CONT. ON FIG. 10b)

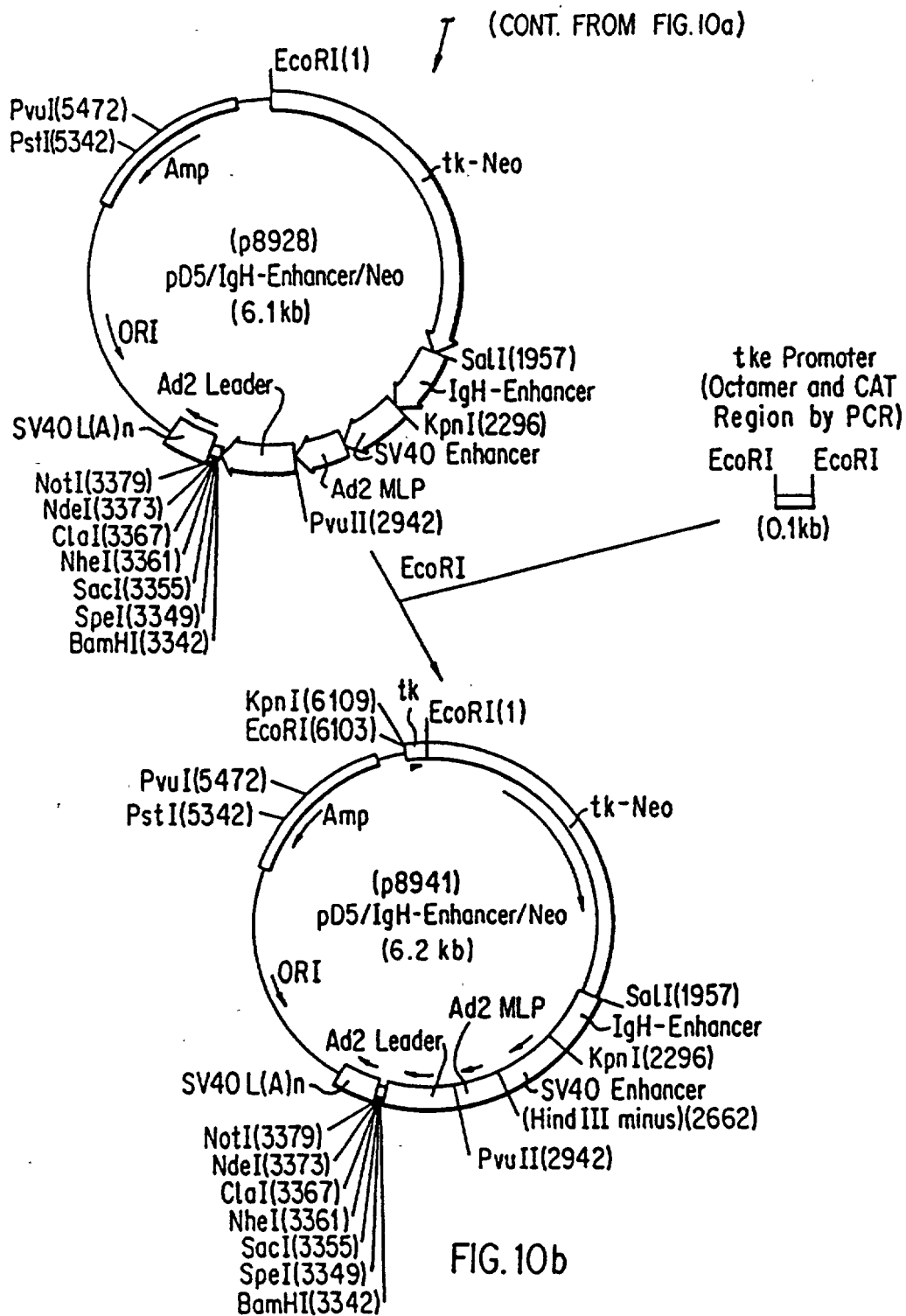


FIG. 10b



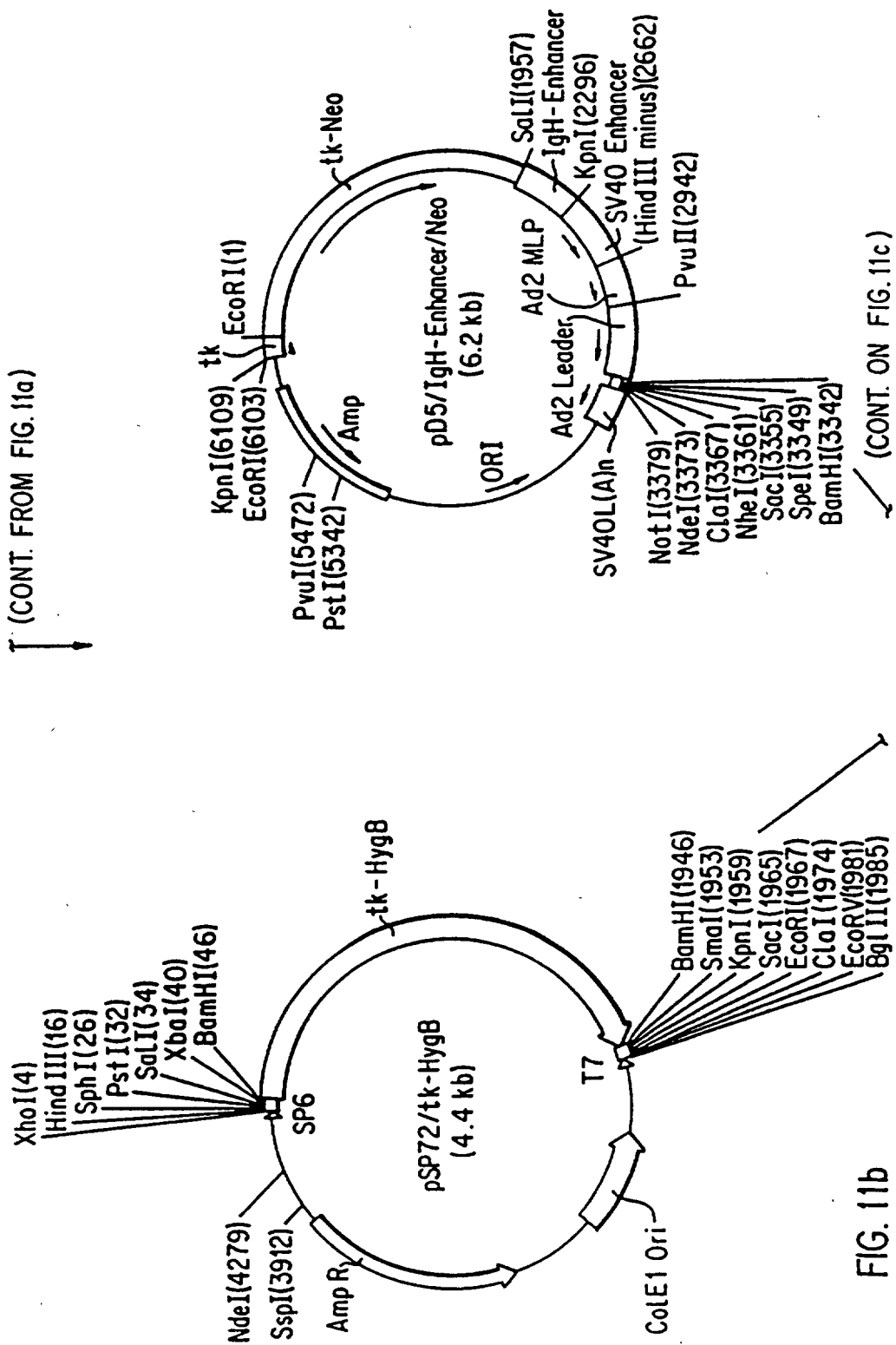


FIG. 11b

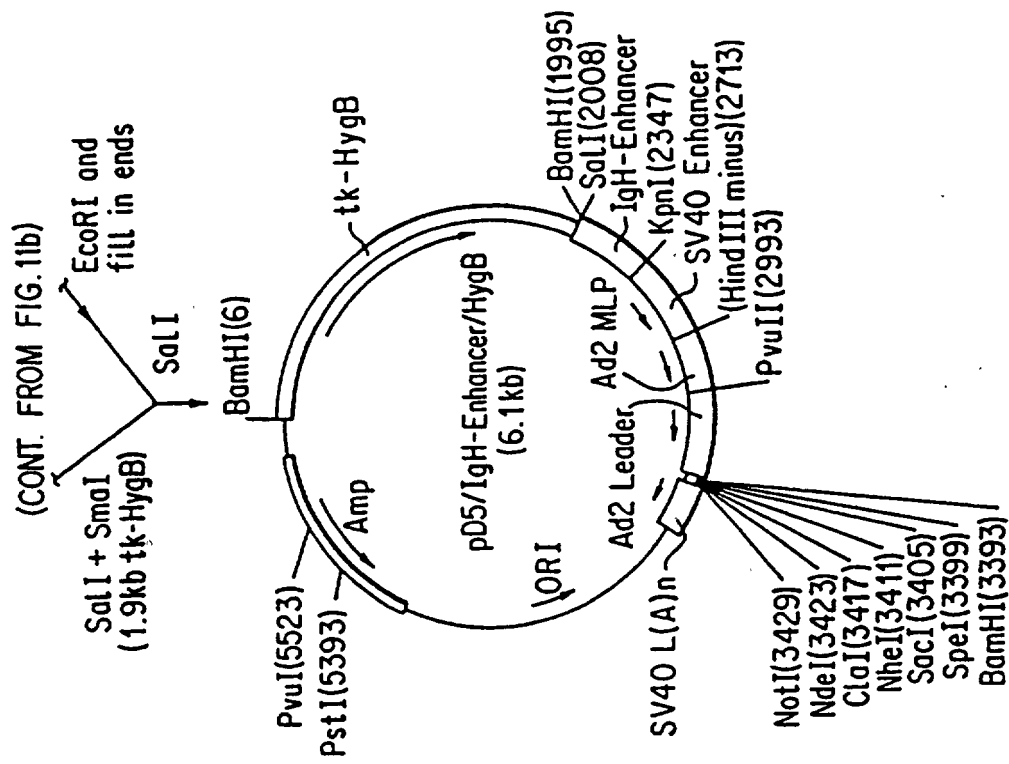


FIG. 11c

Fig. 12

HEAVY AND LIGHT CHAIN VARIABLE REGION FRAMEWORKS

Heavy Chain

v1B4: DVKLVESGGDLVKPGGSLKLSCAASGFTFS [DYYMS] WVRQAP
 m1B4: DVKLVESGGDLVKLGGSLKLSCAASGFTFS [DYYMS] WVRQTP
 Gal: EVQLVESGGDLVQPGRSLRLSCAASGFTFS [BLGMT] WVRQAP

GKGLELVA [AIDNDGGSISYPDTVKG] RFTISRDN SKNTLYLQM
 EKRL ELVA [AIDNDGGSISYPDTVKG] RFTISRDN AKNTLYLQM
 GKGLEWVA [NIKZBGSZZBYVDSVKG] RFTISRDN AKNSLYLQM

NSLRAEDTALYYCAR [-QGRLRRDYFDY] WGQGTLLTVSS...
 SSLRSEDALYYCAR [-QGRLRRDYFDY] WGQGTLLTVSS...
 NSLRVEDTALYYCAR [-----GWGGGD-] WGQGTLLTVST...

Light Chain

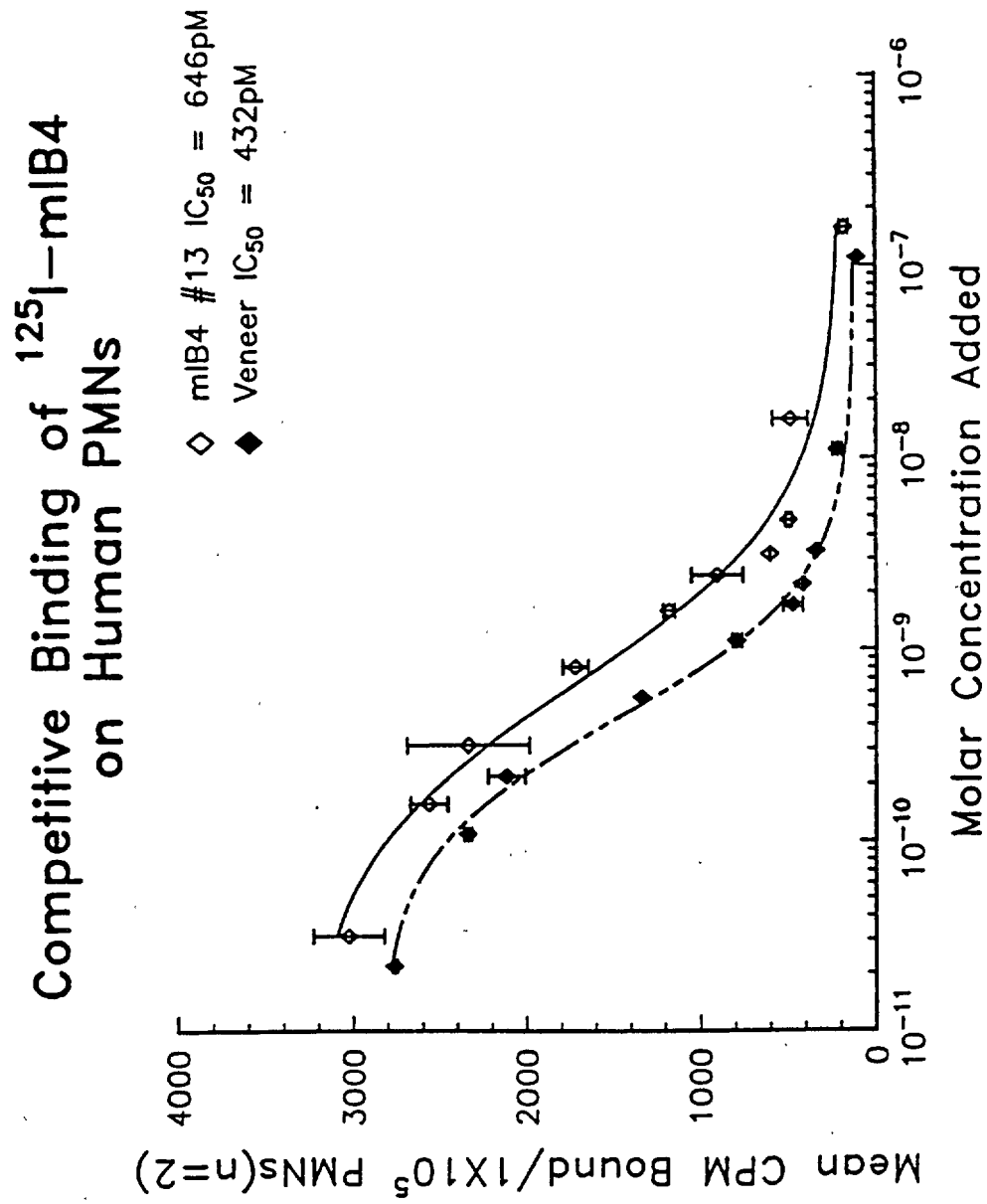
v1B4: DIVMTQSSNSLAVSLGERATISC [RASESVDSYGN SFMH--] WY
 m1B4: DIVLTQSPASLAVSLGQRATISC [RASESVDSYGN SFMH--] WY
 Len: DIVMTQSSNSLAVSLGERATINC [KSSQSVLYSSNSKNYLA] WY

QOKPGQPPKLLIY [RASNLES] GIPDRFSGSGSGTDFTLTISSV
 QOKPGQPPKLLIY [RASNLES] GIPARFSGSGSRTDFTLTINPV
 QOKPGQPPKLLIY [WASTRES] GVPDRFSGSGSGTDFTLTISSL

EADDVATYYC [QQSNEDPLT] FGQGTKLEIKR...
 EADDVATYYC [QQSNEDPLT] FGAGTKLEIKR...
 QAEDVAVYYC [QQYYSTPYS] FGQGTKLEIKR...

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Fig. 13





European Patent
Office

EUROPEAN SEARCH REPORT

Application Number

EP 92 30 4225

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 88, no. 10, 15 May 1991, WASHINGTON DC, US pages 4181 - 4185 S. GORMAN ET AL. 'Reshaping a therapeutic CD4 antibody.' * the whole document *	1-12	C12N15/13 C12P21/08
D,Y	NUCLEIC ACIDS RESEARCH vol. 19, no. 9, 11 May 1991, LONDON, GB pages 2471 - 2476 B. DAUGHERTY ET AL. 'Polymerase chain reaction facilitates the cloning, CDR-grafting, and rapid expression of a murine monoclonal antibody directed against the CD18 component of leukocyte integrins.' * the whole document *	1-12	
D,Y	NATURE. vol. 332, 24 March 1988, LONDON, GB pages 323 - 327 L. RIECHMANN ET AL. 'Reshaping human antibodies for therapy.' * the whole document *	1-12	TECHNICAL FIELDS SEARCHED (Int. Cl.5) C07K
P,X	EP-A-0 438 312 (MERCK & CO., INC.) * claims *	1-12	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 30 SEPTEMBER 1992	Examiner NOOIJ F.J.M.
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

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PCT

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁵ : C12P 21/02, A61K 39/44 C07H 21/00, C07K 13/00 C12N 1/00, 1/21, 5/10 C12N 15/10, 15/63</p>	<p>A1</p>	<p>(11) International Publication Number: WO 93/05168 (43) International Publication Date: 18 March 1993 (18.03.93)</p>
<p>(21) International Application Number: PCT/US92/07066 (22) International Filing Date: 21 August 1992 (21.08.92) (30) Priority data: 755,949 6 September 1991 (06.09.91) US (71) Applicant: RESEARCH DEVELOPMENT FOUNDATION [US/US]; 402 North Division Street, Carson City, NV 89703 (US). (72) Inventors: ROSENBLUM, Michael, G. ; 8810 North Rylander Circle, Houston, TX 77071 (US). BEATTIE, Kenneth, L. ; 2 Hollymeade Drive, The Woodlands, TX 77381 (US).</p>	<p>(74) Agent: WEILER, James, F.; One Riverway, Suite 1560, Houston, TX 77056 (US). (81) Designated States: AU, CA, FI, JP, KR, NO, RU, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE). Published <i>With international search report.</i> <i>With amended claims.</i></p>	
<p>(54) Title: DNA SEQUENCES ENCODING GELONIN POLYPEPTIDE</p> <p>(57) Abstract</p> <p>The present invention provides the nucleotide sequence for a synthetic gene for the plant toxin gelonin and a process for producing, cloning and expressing this synthetic gene. The DNA sequence for a synthetic gene for gelonin as shown in sequence ID No. 1. The present invention also provides expression vectors containing the DNA sequences for gelonin and cells transformed with these vectors. In addition, an immunotoxin comprising an antibody conjugated to the protein gelonin.</p>		

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DNA SEQUENCES ENCODING GELONIN POLYPEPTIDEBACKGROUND OF THE INVENTION1. Field of the Invention

5 This invention relates generally to the immunotoxin gelonin and, more specifically, to the molecular biology of gelonin, including a process for the production of a synthetic gene for gelonin.

2. Description of the Related Art

10 One current interest in cytotoxic substances involves their potential use to specifically target tumor cells. The plant toxin gelonin has received such consideration. Gelonin is a glycoprotein (M.W. approximately 29-30,000 Kd) purified from the seeds of Gelonium multiflorum. Gelonin belongs to a class of potent ribosomal-inactivating plant toxins. Other members of this class of ribosomal-inactivating plant toxins are the chains of abrin, ricin and modeccin. Gelonin, like abrin and ricin, inhibits protein synthesis by damaging the 60S sub-unit of mammalian ribosomes. Although the A chain of ricin (RTA) has been popular for use in immunotoxins, gelonin appears to be more stable to chemical and physical treatment than RTA (Barbieri et al., Cancer Surv. 1: 489-520 (1982)). Furthermore, gelonin itself does not bind to cells and, therefore is non-toxic (except in high concentrations) and is safe to manipulate in the laboratory. 15 20 The inactivation of ribosomes is irreversible, does not appear to involve co-factors and occurs with an efficiency which suggests that gelonin acts enzymatically.

25 Gelonin and ricin are among the most active toxins which inhibit protein synthesis on a protein weight basis. Gelonin is 10 to 1000 times more active in inhibiting protein synthesis than ricin A chain. Peptides like ricin and abrin are composed of two chains, a A chain which is the toxic unit and a B chain which acts by binding to cells. Unlike ricin and abrin, gelonin is composed of a

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single chain, and, because it lacks a B chain for binding to cells, it is itself relatively non-toxic to intact cells (Stirpe, et al. J. Biol. Chem. 255: 6947-6953 (1980)). Mammalian cells apparently lack the ability to bind and/or to internalize the native gelonin molecule. Conjugates of gelonin with a tumor-targeting monoclonal antibody, such as the monoclonal antibody ZME directed to an antigen present on certain tumor cells such as melanoma cells, provide both a specific method for binding the gelonin to the cell and a route for internalization of the gelonin antibody complex. One of the advantages of using the toxin gelonin over using toxins such as ricin A chain is this reduced toxicity to normal tissues as compared to the ricin A chain. Gelonin-coupled with a monoclonal antibody directed to an anti-tumor associated antigen is an active and selective immunotoxic agent for tumor therapy.

Several investigators have utilized gelonin as a cytotoxic agent chemically attached to monoclonal antibodies or to peptide hormone cellular targeting ligands. However, chemical modification of gelonin and cellular targeting moieties can reduce targeting efficiently and cytotoxic potential of gelonin itself. Furthermore, natural sources of gelonin are subject to variability in harvesting and plant growth which can affect gelonin cytotoxic activity. The ability to produce a synthetic gelonin toxin, chemically or utilizing recombinant technology, provides a plentiful, reproducible source of the toxin. Therefore, it is highly desirable to prepare a synthetic gene for gelonin and methods for preparation of the synthetic gene using recombinant technology.

SUMMARY OF THE INVENTION

The present invention provides the nucleotide sequence for a synthetic gene for gelonin and methods for its preparation. The DNA sequence for a synthetic gene for gelonin is shown in SEQ ID NO:1. The present invention also provides expression vectors containing these DNA sequences and cells transformed with these vectors.

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According to one embodiment of the present invention, there is provided a method for producing, cloning and expressing a synthetic gene encoding gelonin. Initially, a double-stranded DNA fragment encoding the primary amino acid sequence determined from purified gelonin protein was designed. This DNA fragment was manipulated to facilitate synthesis, cloning, expression or biochemical manipulation of the gene. Next, a set of synthetic oligonucleotides capable of being linked together to assemble the desired gene was designed, synthesized, purified and 5'-phosphorylated. Subsequently, these oligonucleotides were annealed and ligated together to assemble the intact gene. The synthetic gene was ligated together with a suitable cloning vector and then the nucleotide sequence of the cloned gene was determined. After site directed mutagenesis was conducted to correct any undesired mutations in the cloned gene, the gene is subcloned into a suitable expression vector. The expression vector bearing the synthetic gelonin gene was inserted into a suitable expression host. Thereafter the expression host is maintained under conditions suitable for production of the gelonin gene product and gelonin protein is isolated and purified from cells expressing the gene.

According to another embodiment of the present invention, there is provided a synthetic DNA containing the sequence of nucleotides and fragments and derivatives thereof, coding for protein gelonin or for a polypeptide which inhibits cellular protein synthesis but does not bind a cell surface receptor, with the nucleotide sequence having the formula of Sequence ID NO. 1. Also provided is an expression vector containing synthetic gelonin DNA and host cells containing and expressing the synthetic gelonin gene.

According to yet another embodiment of the present invention, there is provided an immunotoxin comprising an antibody conjugated to the plant toxin protein gelonin.

For the purposes of the present invention, the term "fragment" is defined as any portion of SEQ ID No. 1 that would produce a protein that inhibits

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cellular protein synthesis but does not bind to a surface receptor. "Derivative" is defined as having the substitution of one or more individual nucleic acids such that the same protein or polypeptide is produced.

5 The present invention recognizes and addresses the previously mentioned long felt needs and provides a satisfactory meeting of those needs and its various embodiments. To one of skill in this art who has the benefit of this invention's teachings and disclosures, other and further objects and advantages will be clear, as well as others inherent therein. In conjunction with the accompanying drawings, the following description of presently preferred
10 embodiments are given for the purpose of disclosure. Although these descriptions are detailed to insure adequacy and aid understanding, this is not intended to prejudice the purpose of a patent which is to claim an invention no matter how others may later disguise it by variations and form or additions of further improvements.

15 **BRIEF DESCRIPTION OF THE DRAWINGS**

So that the manner in which the above recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain
20 embodiments thereof which are illustrated in the appended drawings. These drawings form a part of this specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting of its scope. The invention may admit to other equally effective equivalent embodiments.

25 FIG. 1 depicts the schematic illustration of a process for chemically synthesizing oligonucleotides.

FIG. 2 shows an illustration of the solid phase gene assembly process.

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FIG. 3 illustrates a schematic diagram of the gelonin gene assembly process.

FIG. 4 shows the schematic illustration of the arrangement of oligonucleotides in the gelonin gene assembly.

FIG. 5 depicts the oligonucleotide sequences used to assemble the gelonin gene.

DETAILED DESCRIPTION OF THE INVENTION

Recent developments in molecular biology have enabled the cloning, expressing and genetic engineering of numerous genes encoding proteins of biomedical and agricultural importance. A significant recent advance in this field is the capability to design and produce synthetic genes. The synthetic genes can encode proteins of known amino acid sequence, as well as novel proteins not existing in nature. This is particularly useful in the case of proteins of therapeutic value, such as gelonin. It is feasible to design, synthesize, clone and express a gene based on the amino acid sequence of a protein. The design, sequence, cloning, and expression are also possible even if specific information about the natural gene is unavailable, for example, if the gene has not yet been cloned. Furthermore, gene synthesis facilitates the engineering of variant gene products, possessing properties not found in the naturally occurring protein. For example, a gene encoding a protein normally found only in plants or animals can be designed, synthesized, and cloned into a vector which can yield large quantities of the protein in microbial hosts cells.

One advantage of synthetic genes is related to the redundancy of the genetic code. Most amino acids can be encoded by more than one three-base "codon" within a gene. Different organisms tend to employ different sets of codons for their proteins (S. Aota et al., Nucleic Acids Res., v. 16, Supplement, pp. r315-r391 (1987). In other words, the codons that are "preferred" by one organism are different from those preferred by another organism. This phenomenon is believed to be related to differences in the relative abundance of

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specific iso-accepting transfer RNA molecules for a given amino acid. Synthetic genes can be designed to incorporate preferred codons for a given expression system, even if the gene product is from a "heterologous" host organism, which employs a different set of preferred codons for some amino acids. It has been shown that synthetic genes encoding mammalian proteins can yield significantly greater protein product in a microbial host if the codons chosen in the gene design correspond to those most commonly used by the microbial host. This phenomenon was considered in the design of the gelonin gene (originally from plants) intended for introduction into the Escherichia coli expression host. For expression in a different host system the gelonin gene could be easily redesigned by one having ordinary skill in this art to incorporate codons optimal for the different host.

Another advantage of synthetic genes, actually enabled by the redundancy of the genetic code in this case, is that the DNA sequence encoding the gene of interest can be modified (without changing the amino acid sequence encoded) to contain a maximum number of uniquely occurring restriction enzyme recognition sites across the gene. The existence of numerous single-cutting restriction sites along a gene greatly facilitates the biochemical manipulation of the gene. For example, the ability to cleave out a segment of a gene and replace it with a different DNA sequence (enabled by closely spaced unique restriction sites) facilitates the introduction of new genetic information into the gene by recombinant DNA techniques. Useful manipulations enabled by this approach include introduction of mutations into defined regions of a gene, correction of mutations arising during gene synthesis, assembly and cloning, and creation of chimeric or fusion genes encoding proteins that combine functional domains of separate proteins.

The design of a synthetic gene can also accommodate non-coding "flanking" DNA sequences which can facilitate cloning and expression of the gene. For example, restriction endonuclease recognition sequences can be

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incorporated into flanking regions of the gene to enable the specific ligation of the gene into the desired cloning vector. Additionally, genetic signals can be incorporated into a synthetic gene which serve to control gene expression in vivo.

In addition, the de novo gene design can enable the in vivo targeting of a gene product to a certain tissue or organelle, which can enhance the therapeutic action of the gene product. For example, it is feasible to target a therapeutic agent to a particular cell type by incorporating into the relevant gene a sequence which encodes a polypeptide domain that specifically binds to a receptor on the surface of target cells.

Synthetic gene design can furthermore include considerations that are related to the chemical synthesis of short DNA strands and the assembly of oligonucleotides into longer duplex DNA segments. It is advantageous in the chemical synthesis of DNA to avoid the poor coupling efficiency associated with consecutive addition of numerous G or C residues to a chain. Also, it is desirable to avoid DNA sequences that contain intrastrand secondary structure (hairpin structure) or intermolecular complementarity, which can interfere with correct assembly of a gene during the annealing of component oligonucleotides. These objectives can usually be achieved by choosing alternative codons in the gene design.

The process of gene assembly comprises the following steps. First, the nucleotide sequence corresponding to the two strands of the desired coding region are written out, providing for perfect complementary base pairing between the two strands. Then any desired flanking sequences are added. For example, flanking sequences can be added to incorporate restriction endonuclease recognition sites adjacent to the coding sequence. The gene is then divided into overlapping sets of single-stranded fragments. The single-stranded fragments are chemically synthesized by automated DNA synthesis instruments. The extent of complementary overlap between successive oligonucleotides along the synthetic gene is a matter of choice, but is typically 6-20 bases. After all of the

oligonucleotides needed for the synthetic gene have been chemically synthesized, they are preferably purified by polyacrylamide gel electrophoresis or high performance liquid chromatography. The purified oligonucleotides are then 5'-phosphorylated by the action of polynucleotide kinase and adenosine 5'-triphosphate. The strands are then annealed together, either in a single mixture, in blocks of 3-10 overlapping oligonucleotides, or by stepwise addition of oligonucleotides on a solid phase support. The ends of the assembled gene are provided with restriction sites which are employed in cloning of the gene. The assembled gene is typically cloned initially into the single-stranded vector M13 for convenient DNA sequencing. If necessary, mutations are corrected by oligonucleotide-directed mutagenesis.

Desired features of the synthetic gene (optimal codon usage, occurrence of unique restriction sites, elimination of secondary structure, etc.) can be designed with the assistance of any of several commercially available DNA sequence analysis programs for microcomputers.

Two recent developments, both utilized in the instant invention, enable genes to be synthesized more rapidly and economically and create new opportunities for protein engineering (K. L. Beattie et al., *Biotechnol. Appl. Biochem.*, 10, 510-521 (1988); K. L. Beattie and R. F. Fowler, *Nature*, 352, 548-549 (1991)). The first development, illustrated in Figure 1, is a technology for rapid, economical synthesis of large numbers of oligonucleotides. This technology enables the preparation of all of the synthetic DNA needed for assembly of a gene in a single day. Referring to Fig. 1, nucleoside-derivatized controlled pore glass is placed within individual synthesis wafers, consisting of Teflon cylinders with porous ends to allow fluid flow through a stack of the wafers. Simultaneous addition of A, G, C or T to DNA chains attached to the CPG and retained within the wafers is accomplished by the sequential flow of reagents through the columns by the phosphoramidite method (L. J. McBride and M. H. Caruthers, *Tetrahedron Lett.*, 24, 245-248 (1983)). After completion of

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each chemical reaction cycle, the wafers are sorted into different columns to provide for synthesis of a different nucleotide sequence within each wafer. The sequential position of one wafer (darkened) through four cycles is depicted, which would result in the addition of AGCT to the growing DNA chain contained therein.

A second technology development which facilitates gene synthesis provides a means for stepwise joining of synthetic oligonucleotides on a solid phase support to form a gene. Referring to Fig. 2, the desired gene is designed to be assembled from a set of overlapping complementary oligonucleotides. Assembly is initiated with an oligonucleotide bound at one end to a solid phase support. 5'-phosphorylated oligonucleotides are added sequentially (at molar excess) to the support-bound-strand. At each step of the gene assembly unbound DNA is washed away before the next annealing reaction is performed. The completed assembly is treated with DNA ligase to seal the nicks, then the gene is released from the support by cleavage at a unique restriction site contained within the support-bound oligonucleotide. The released DNA is ligated into a suitable vector for sequencing and expression.

Numerous options exist for vector-host environments in the expression of the synthetic gene resulting in production of the encoded protein. These are discussed in detail in Methods in Enzymology, Vol. 152, 1987, Academic Press. Briefly, specialized expression vectors are available for insertion into bacterial, fungal, animal or plant hosts. The bacterium Escherichia coli is most commonly used for expression of "foreign" genes. The yeast Saccharomyces cerevisiae is another popular expression host. As mentioned previously, if the cloned gene of interest is chemically synthesized, optimal codon usage for the desired expression host is provided for in the gene design to increase the level of expression. Most expression vectors contain genetic control elements positioned adjacent to the cloning site which drive high levels of gene expression. Inducible promoters of expression vectors are typically derived from bacteria

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(e.g., tac, trp) or viruses (e.g., lambda, SV40). "Signal sequence" elements are sometimes included in the vector, to direct transport of the gene product out of the host cell. The signal sequence elements can facilitate purification and reduce proteolytic degradation. In the case of synthetic genes any desired genetic control element can be included within the duplex DNA being assembled. Some expression vectors contain a coding sequence immediately adjacent to the cloning site, such that in-frame insertion of the foreign coding sequence results in production of a fusion protein. The additional coding sequence can be provided within the assembled sequence in the case of synthetic genes. The production of a gene as a fusion protein can provide several benefits, including increased expression, greater stability, fast affinity purification using a support-bound ligand that binds to the additional polypeptide component, and cellular targeting of the gene product (for example, to a cell type possessing a cell surface receptor for the additional polypeptide component). Gene engineering incorporating this latter feature may be employed in the further development of gelonin-based therapeutics.

In the cloning and expression of DNA sequences encoding the plant toxin gelonin a wide variety of vectors are useful. These include, for example, vectors consisting of segments of chromosomal, non-chromosomal and synthetic DNA sequences, such as various known derivatives of SV40, known bacterial plasmids, (e.g.), plasmids from E.coli including col E1, pCR1, pBR322, pMB9 and their derivatives, wider host range plasmids, (e.g.), RP4, phage DNAs, (e.g.), the numerous derivatives of phage lambda, (e.g.), NM 989, and other DNA phages, (e.g.), M13 and filamentous single stranded DNA phages, yeast plasmids such as the 2 mu plasmid or derivatives thereof, and vectors derived from combinations of plasmids and phage DNAs, such as plasmids which have been modified to employ phage DNA or other expression control sequences.

Within each specific cloning or expression vehicle, various sites may be selected for insertion of the DNA sequences of this invention. These sites

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are usually designated by the restriction endonuclease which cuts them and are well recognized by those of skill in the art. Various methods for inserting DNA sequences into these sites to form recombinant DNA molecules are also well known. These include, for example, dG-dC or dA-dT tailing, direct ligation, 5 synthetic linkers, exonuclease and polymerase-linked repair reactions followed by ligation, or extension of the DNA strand with DNA polymerase and an appropriate single-stranded template followed by ligation. It is, of course, to be understood that a cloning or expression vehicle useful in this invention need not have a restriction endonuclease site for insertion of the chosen DNA fragment. 10 Instead, the vehicle could be joined to the fragment by alternative means.

For expression of the DNA sequences of this invention, these DNA sequences are operatively-linked to one or more expression control sequences in the expression vector. Such operative linking, which may be effected before or after the chosen DNA sequence is inserted into a cloning vehicle, enables the 15 expression control sequences to control and promote the expression of the inserted DNA sequence.

Any of the wide variety of expression control sequences that control the expression of a DNA sequence may be used in these vectors to express the DNA sequence of this invention. Such useful expression control sequences, 20 include, for example, the early and late promoters of SV40, the lac system, the trp system, the TAC or TRC system, the major operator and promoter regions of phase lambda, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast alpha-mating factors, and 25 other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. In mammalian cells, it is additionally possible to amplify the expression units by linking the gene to that coding for dehydrofolate reductase and applying a selection to host Chinese hamster ovary cells.

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The vector or expression vehicle, and in particular the sites chosen therein for insertion of the selected DNA fragment and the expression control sequence employed in this invention are determined by a variety of factors. For example, these factors include the number of sites susceptible to a particular restriction enzyme, size of the protein to be expressed, expression characteristics such as the location of start and stop codons relative to the vector sequences. Other factors will be recognized by those of skill in the art. The choice of a vector, expression control sequence, and insertion site for a particular phospholipase inhibitor protein sequence is determined by a balance of these factors with not all selections being equally effective for a given case.

The recombinant DNA molecule containing the desired gene operatively linked to an expression control sequence may then be employed to transform a wide variety of appropriate hosts so as to permit such hosts (transformants) to express the gene, or fragment thereof, and to produce the polypeptide, or portion thereof, for which the hybrid DNA codes.

A wide variety of hosts are also useful in producing the antigens and DNA sequences of this invention. These hosts include, for example, bacteria, such as E.coli, Bacillus and Streptomyces, fungi, such as yeasts, and animal, such as CHO cells, and plant cells in tissue culture. The selection of an appropriate host is controlled by a number of factors recognized by the art. These include, for example, compatibility with the chosen vector, toxicity of the co-products, ease of recovery of the desired polypeptide, expression characteristics, biosafety and costs. No absolute choice of host maybe made for a particular recombinant DNA molecule or polypeptide from any of these factors alone. Instead, a balance of these factors must be struck with the realization that no all hosts may be equally effective for expression of a particular recombinant DNA molecule.

As we have previously demonstrated, it should be understood that the DNA sequences that are inserted at the selected site of a cloning or expression vehicle may include nucleotides which are not part of the actual gene coding for

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the desired polypeptide or may include only a fragment of the entire gene for that protein. It is only required that whatever DNA sequence is employed, the transformed host produces a protein gelonin or a polypeptide having substantially the same functional activity as gelonin. For example, the DNA sequences of this invention may be fused in the same reading frame in an expression vector to a portion of a DNA sequence coding for at least one eukaryotic or prokaryotic or prokaryotic carrier protein or a DNA sequence coding for at least one eukaryotic or prokaryotic signal sequence, or combinations thereof. Such constructions may aid in expression of the desired DNA sequence, improve purification or permit secretion, and preferably maturation, of the desired polypeptide from the host cell. The DNA sequence may alternatively include an ATG start codon, alone or together with other codons, fused directly to the sequence encoding the first amino acid of a desired polypeptide. Such constructions enable the production of, for example, a methionyl or other peptidyl polypeptide, that is part of this invention. This N-terminal methionine or peptide may then be cleaved intra- or extracellularly by a variety of known processes or the polypeptide used together with the methionine or other fusion attached to it in the compositions and methods of this invention.

EXAMPLES

Synthesis & Assembly of Gelonin Gene

Example 1

Binding of 5'-biotinylated oligonucleotide to streptavidin-coated latex microspheres:

A 0.2 ml sample of DYNABEADS M280 Streptavidin (Dynal Corp.) was placed into a 1.5 ml Eppendorf tube. The tube was held against a magnetic plate (Advanced Magnetix, Inc.) for a few minutes to cause the paramagnetic latex microspheres to be drawn to the side of the tube, then the fluid was drawn off. The beads were washed twice with 0.2 ml annealing buffer

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(composition given below) at room temp., then resuspended in 0.2 ml annealing buffer.

To the bead suspension was added 1 nmol of 5'-biotinylated oligonucleotide. After 30 min. at room temperature the beads were washed twice with 0.2 ml annealing buffer and resuspended in 0.2 ml annealing buffer. Spectrophotometric analysis of the unbound oligonucleotide in the washes indicated that approximately 300 pmol of oligonucleotide were bound to the beads.

Example 2

Annealing/washing cycle, repeated for addition of each successive oligonucleotide:

Before use in the gene assembly oligonucleotides were purified by polyacrylamide gel electrophoresis and enzymatically 5'-phosphorylated using T4 polynucleotide kinase.

To 150 pmol support-bound oligonucleotide were added 750 pmol overlapping complementary oligonucleotide and the annealing was carried out in 0.10 ml 50mM sodium phosphate buffer, pH 7.5, 1M NaCl (annealing buffer) for 5 min. at 45 deg. C, then the mixture was cooled to room temperature over a 7 min. period. Beads were then washed twice with 0.2 ml of the same buffer at room temperature. This cycle was repeated until the last oligonucleotide in the assembly was added.

Example 3

Ligation of product and release from the support by restriction enzyme digestion:

After completion of the assembly the beads were washed and resuspended in 0.04 ml ligase buffer. After addition of 0.005 ml DNA ligase (New England Biolabs, high specific activity grade) the mixture was incubated at room temperature for 2 hours then washed and resuspended in 0.04 ml restriction digestion buffer. After addition of ten units of restriction endonuclease EcoRI the mixture was incubated at 37 degrees C for 90 minutes. The liquid was drawn off

-15-

and the released DNA was ethanol precipitated and resuspended in 0.01 ml ligase buffer.

Example 4

Assembly of the gelonin gene:

5 The gene was assembled from both directions as illustrated in FIG 3 and FIG 4. The oligonucleotide sequences are identified in Figure 5. Assembly of the 5'-end of the gene (approximately 500 bp N-terminal coding region) began with support-bound Btgel1 and the oligonucleotides were added in the following order (each involving one annealing/washing cycle): gel1, gel38, gel2, gel37, gel3, gel36, gel4, gel35, gel5, gel34, gel6, gel33, gel7, gel32, 10 gel8, gel31, gel9, gel30, gel10, gel29, gel11, gel28, gel12, gel27.

Assembly of the 3'-end of the gene (approximately 300 bp C-terminal coding region) began with support-bound Btgel2 and the oligonucleotides were added in the following order: gel20, gel19, gel21, gel18, gel22, gel17, 15 gel23, gel16, gel24, gel15, gel25, gel14, gel26, gel13.

Referring to FIG. 3, the 5'-end of the gene (N-terminal coding region) was released from the support by digestion with restriction endonuclease EcoRI, and the 3'-end of the gene (C-terminal coding region) was released from the support by digestion with restriction endonuclease HindIII. Referring to FIG. 20 4, the two gene fragments, containing complementary 20-base tails within oligonucleotides gel27 and gel13, were annealed together, then ligated to form the intact gene.

Example 5

Cloning of the synthetic gelonin gene:

25 The completed DNA product was ligated with M13mp19RFDNA that had been cleaved with EcoRI and HindIII, according to standard methods described in Molecular Cloning: A laboratory manual, E.F. Sambrook et al., 1989:

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Example 6Sequencing of the synthetic gelonin gene:

The sequence of the synthetic gene in M13mp19 was confirmed by dideoxy sequencing. Two mutations were found in the cloned synthetic gene, both in the 5'-end (N-terminal coding region).

Example 7Site-directed mutagenesis to correct mutations in cloned synthetic gene:

Oligonucleotide-directed mutagenesis was carried out to correct the two mutations within the gelonin gene, following the procedure supplied with the in vitro mutagenesis reagent kit (Amersham Corp.).

Example 8Subcloning of synthetic gene into expression vector:

The synthetic gelonin gene was cleaved from the M13mp19 vector by action of EcoRI and HINDIII and the gene-containing fragment was purified by agarose gel electrophoresis and ligated into EcoRI/HindIII-cleaved expression vector pKK223-3 (Pharmacia).

Example 9Analysis of expression of synthetic gelonin gene in E. coli:

A 50 ml culture of E. coli JM105 bearing the synthetic gelonin gene cloned into pKK223-3 is grown up, induced with IPTG and lysed to obtain a crude extract. The extract is analyzed by SDS polyacrylamide gel electrophoresis (along side a control extract prepared from host cells carrying the expression vector with no insert). Western blot analysis and functional assays of gelonin are also conducted to confirm that the protein is expressed and active.

In conclusion, therefore, it is seen that the present invention and the embodiments disclosed herein are well adapted to carry out the objectives and obtain the ends set forth at the outset. Certain changes can be made in the method and apparatus without parting from the spirit and scope of this invention. It is realized that changes are possible and it is further intended that each element

[illegible]

SEQUENCE LISTING

5

- (ii) TITLE OF INVENTION: DNA SEQUENCES ENCODING GELONIN
POLYPEPTIDE

(111) NUMBER OF SEQUENCES: 1

(iv) CORRESPONDENCE ADDRESS:

10

- (A) ADDRESSEE: James F. Weiler, Attorney-at-Law
(B) STREET: One Riverway, Suite 1560
(C) CITY: Houston.
(D) STATE: Texas
(E) COUNTRY: USA
(F) ZIP: 77056

15.

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WordPerfect 5.1

20

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: US 07/755,949
(B) FILING DATE: 06-SEP-1991
(C) CLASSIFICATION:

25

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Weiler, James F.
(B) REGISTRATION NUMBER: 16,040
(C) REFERENCE/DOCKET NUMBER: D-5385

(ix) TELECOMMUNICATION INFORMATION:

30

- (A) TELEPHONE: (713) 626-8646
(B) TELEFAX: (713) 963-5853

(2) INFORMATION FOR SEQ ID NO:1:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 783 base pairs

[illegible]

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(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

5 (iii) HYPOTHETICAL: YES

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Gelonium multiforum*

(D) DEVELOPMENTAL STAGE: Seed

(F) TISSUE TYPE: Nut

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGGGTCTGG ATACCGTTAG CTTCAGCACC AAAGGCGCGA CCTACATCAC CTACGTTAAC	60
TTCTGAAACG AACTGCGTGT TAAACTGAAA CCGGAAGGTA ACAGCCATGG CATCCCCTG	120
CTGCGTAAAG GTGATGACCC GGGTAAATGC TTCGTGCTGG TGGCGCTGAG CAACGATAAC	180
GGTCAGCTGG CAGAAATCGC AATCGATGTT ACCAGCGTGT ACGTAGTTGG CTATCAGGTG	240
CGTAACCGCA GCTACTTCTT CAAAGATGCT CCGGATGCAG CGTACGAAGG CCTGTTCAAA	300
AACACCATCA AAAACCCGCT GCTGTTCCGT GGCAAAATC GTCTGCACTT CCGTGGCAGC	360
TATCCGAGCC TCGAAGCCGA AAAAGCGTAC CGCGAAACTA CCGATCTGGG TATCGAACCG	420
CTGCGCATCG GCATCAAAAA ACTGGACGAA AACGCGATCG ACAACTACAA ACCGACCGAA	480
ATCGCGAGCT CTCTGCTGGT TGTGATCCAG ATGGTGAGCG AAGCGGCACG TTTCACCTTC	540
ATCGAAAACC AGATTTCGTAA CAACTTCCAG CAGCGTATCC GTCCGGCGAA CAACACCATC	600
TCTCTGGAAA ACAATGGGG CAACTGAGC TTCCAGATCC GTACCAGCGG TGCGAACGGT	660
ATGTTCAAGG AAGCGGTGGA ACTGGAACGC GCGAACGGCA AAAAATACTA CGTGAATCGG	720
GTGGATCAGG TGAAACCGAA AATCGCACTG CTGAAATTCG TGGACAAAGA CCCGGAATAG	780
TGA	783

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CLAIMS

1. A method of producing a synthetic gene encoding gelonin, comprising the steps of:

5 designing a first DNA fragment containing the sequence
coding for gelonin, wherein said sequence contains a three
base codon triplet for each amino acid in the gelonin
sequence, said triplet is selected from the group of triplets
coding for each amino acid to maximize expression,
manipulation or stability of the DNA fragment in an expression
vector synthesizing said first DNA fragment;

10 synthesizing a complementary DNA fragment to said first
DNA fragment; and

15 forming a DNA duplex by annealing said first DNA
fragment and complementary DNA fragment.

2. The method of claim 1, wherein said synthesizing
steps comprise:

20 dividing said DNA fragment into overlapping sets of
single-stranded fragments of oligonucleotides, said fragments
being chemically synthesized;
purifying said oligonucleotides;
phosphorylating said oligonucleotides on the 5' end; and
annealing strands of oligonucleotides together.

25 3. The method of claim 1, wherein flanking sequences
are attached to said duplex DNA.

30 4. The method of claim 1, further comprising the
incorporation of restriction endonuclease recognition sites into
said synthetic gene or into a flanking sequence.

5. A method of cloning the synthetic gene of claim 1
into an expression vector, comprising the steps of:

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(a) isolating the double stranded DNA encoding the gene in linear duplex form;

5 (c) joining said gene-containing linear duplex DNA
with said expression vector; and

10

6. The product of the process of claim 5.

15 (b) growing the host cells containing the gene cloned into the expression vector, under conditions that induce the synthesis of the gene product in acceptable quantities;

(c) purifying the gene product from said cells.

25 9. The method of claim 7 further comprising the step of determining the gene product by SDS gel electrophoresis, Western blot analysis, activity assay or other suitable means.

30 11. The process of claim 1, wherein the triplet codons
are selected to provide expression in a suitable bacterial host.

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13. The product of the process of claim 11.
14. The process of claim 1, wherein the triplet codons are selected to provide expression in a suitable fungal host.
15. The product of the process of claim 14.
16. The process of claim 14, wherein the host is yeast.
17. The process of claim 1, wherein the codons are selected to provide expression in mammalian cells.
18. The product of the process of claim 17.
19. The process of claim 1, wherein the codons are selected to provide expression in insect cells.
20. The product of the process of claim 19.
21. The process of claim 1, wherein the codons are selected to provide expression in plant cells.
22. The product of the process of claim 21.
23. The process of claim 1, wherein all of the oligonucleotides are annealed together in one reaction mixture to assemble the gene.
24. The process of claim 1, wherein the oligonucleotides are annealed in blocks, said blocks are subsequently annealed and ligated together to form the intact gene.

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25. The process of claim 1, wherein the gelonin gene is constructed by a solid phase assembly process comprising the steps of:

- 5 a. attaching a synthetic oligonucleotide at or near one of its ends to a solid phase support material;
- b. washing away excess, unbound oligonucleotide;
- c. adding a molar excess of oligonucleotide, which possesses perfect base sequence complementarity with the support-bound oligonucleotide, throughout part or all of its
10 length, and annealing the strands together to form a duplex DNA structure, then washing away excess, unbound oligonucleotide;
- d. repeating step c until the entire gene has been assembled;
- 15 e. treating the assembled gene with DNA ligase to form an intact duplex DNA; and
- f. releasing the synthetic gene from the support with a restriction enzyme or other suitable means.

20 26. Synthetic DNA containing a sequence of nucleotides coding for a protein gelonin or for a polypeptide which inhibits cellular protein synthesis but does not bind to a cell surface receptor.

25 27. The synthetic DNA of claim 26, wherein said nucleotide sequence is SEQ ID NO. 1 and fragments and derivatives thereof.

30 28. The synthetic DNA sequence of claim 27 wherein said DNA sequence inhibits cellular protein synthesis but does not bind a cell surface receptor.

29. An expression vector containing a DNA of claims 26 or 27.

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30. The vector of claim 29, wherein said vector is pKK223-3.

5 31. The vector of claim 29, wherein said vector is pKC30 or one of its derivatives.

32. A host cell containing and capable of expressing a recombinant DNA molecule of claims 26 or 27.

10 33. The host of claim 32 wherein said host is selected from the group consisting of bacteria, fungi, insect cells, animal and plant hosts.

15 34. The host of claim 32, wherein said host is selected from the group consisting of strains of E.Coli, Pseudomonas, Bacillus and yeast.

20 35. The host of claim 32 wherein said host is selected from the group consisting of mouse, swine, and human tissue cells.

36. The host of claim 34, wherein the E.Coli is E.Coli JM105.

25 37. The synthetic DNA of claim 27, wherein said DNA sequence is operatively linked to an expression control sequence.

30 38. The synthetic DNA of claim 37 wherein said expression control sequence controls the expression of genes of prokaryotic or eukaryotic cells or their viruses.

39. The synthetic DNA of claim 37, wherein said expression control sequence is selected from the group consisting of an early promoter SV40, a late promoter SV40, a lac system, a TAC system, a TRC system, a TRP system, major operator and promoter

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b. designing, synthesizing, purifying, the 5'-phosphorylating a set of synthetic oligonucleotides which are

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capable of being linked together to assemble the synthetic gene;

c. annealing and ligating the oligonucleotides together to assemble the synthetic gene;

5

43. An immunotoxin comprising:

(a) the product of the process of claim 42 and

(b) an antibody conjugated to said product.

10

44. A process for producing, cloning and expressing a synthetic gene encoding gelonin, comprising the steps of:

a. designing a double-stranded DNA fragment containing a sequence of three base codons corresponding to the primary amino acid sequence determined from purified gelonin protein so that said DNA sequence has features that facilitate synthesis, cloning, expression, functioning, or biochemical manipulation of the gene;

15

b. designing, synthesizing, purifying, the 5'-phosphorylating a set of synthetic oligonucleotides which are capable of being linked together to assemble the synthetic gene;

20

c. annealing and ligating the oligonucleotides together to assemble the synthetic gene;

d. ligating the synthetic gene together with a suitable cloning vector;

25

e. determining the nucleotide sequence of the cloned gene to verify the correctness of the gene;

f. conducting site-directed mutagenesis to correct any undesired mutations in the cloned gene;

30

g. subcloning the gene into a suitable expression vector;

h. introducing the expression vector bearing the synthetic gelonin gene into a suitable expression host;

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i. growing the expression host bearing the gelonin-expression vector under conditions suitable for production of the gelonin product; and

5 j. isolating and purifying the gelonin from cells expressing the gene.

45. An immunotoxin comprising:

- (a) the product of the process of claim 44; and
- (b) an antibody conjugated to said product.

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46. A method for verifying the accuracy of the assembled and cloned gene of claim 1, further comprising the steps of:

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- (a) isolating vector DNA bearing the gene of claim 1;
- (b) sequencing the gene-bearing vector DNA; and
- (c) repeating steps a and b with a multiplicity of clones until a clone containing the desired DNA sequence has been identified.

20

47. A method for correcting mutations arising in the cloned gene of claim 1 by site-directed mutagenesis, further comprising the steps of:

25

- (a) isolating the DNA of said mutation-containing gene;
- (b) annealing the mutation-containing DNA strand with a synthetic corrective oligonucleotide primer spanning a site of said mutation and containing a correct DNA sequence;
- (c) elongating the corrective oligonucleotide primer annealed to said mutation-bearing template strand by action of a DNA polymerase;
- (d) introducing the DNA product of step c into an appropriate host cell by transfection or transformation;
- (e) isolating vector DNA; and

30

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(f) subcloning the corrected gene into an expression vector.

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[received by the International Bureau on 18 January 1993 (18.01.93);
original claim 38 cancelled; original claims 5,9,11,14,26,28,32,34,36,
37,39,44 and 46 amended; remaining claims unchanged; claims 39-47
renumbered as claims 38-46 (9 pages)]

designing a first DNA fragment containing the sequence
coding for gelonin, wherein said sequence contains a three base
codon triplet for each amino acid in the gelonin sequence, said
triplet is selected from the group of triplets coding for each amino
acid to maximize expression, manipulation or stability of the DNA
fragment in an expression vector synthesizing said first DNA
fragment;

forming a DNA duplex by annealing said first DNA fragment and complimentary DNA fragment.

dividing said DNA fragment into overlapping sets of single-stranded fragments of oligonucleotides, said fragments being chemically synthesized;

purifying said oligonucleotides;
phosphorylating said oligonucleotides on the 5' end; and
annealing strands of oligonucleotides together.

3. The method of claim 1, wherein flanking sequences are attached to said duplex DNA.

4. The method of claim 1, further comprising the incorporation of restriction endonuclease recognition sites into said synthetic gene or into a flanking sequence.

5. A method of cloning the synthetic gene of claim 1 into an expression vector, comprising the steps of:

[illegible]

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- (a) isolating the double stranded DNA encoding the gene
in linear duplex form;
- (b) cleaving an expression vector with a restriction
endonuclease;
- 5 (c) joining said gene-containing linear duplex DNA with
said expression vector.
6. The product of the process of claim 5.
- 10 7. A method of purifying the gene of claim 1, comprising
the step of:
- (a) cloning the gene into an expression vector;
- (b) growing the host cells containing the gene cloned into
the expression vector, under conditions that induce the synthesis
15 of the gene product;
- (c) purifying the gene product from said cells.
8. An immunotoxin comprising:
- (a) the product of the process of claim 7; and
- 20 (b) an antibody conjugated to said product.
9. The method of claim 7 further comprising the step of
determining the gene product by SDS gel electrophoresis, Western blot
analysis and activity assay.
- 25 10. The product of the method of claim 7.
11. The process of claim 1, wherein the triplet codons are
selected to provide expression in a bacterial host.
- 30 12. The process of claim 11, wherein said host is
Escherichia coli.

13. The product of the process of claim 11.

14. The process of claim 1, wherein the triplet codons are selected to provide expression in a fungal host.

5

15. The product of the process of claim 14.

16. The process of claim 14, wherein the host is yeast.

10

17. The process of claim 1, wherein the codons are selected to provide expression in mammalian cells.

18. The product of the process of claim 17.

15

19. The process of claim 1, wherein the codons are selected to provide expression in insect cells.

20. The product of the process of claim 19.

20

21. The process of claim 1, wherein the codons are selected to provide expression in plant cells.

22. The product of the process of claim 21.

25

23. The process of claim 1, wherein all of the oligonucleotides are annealed together in one reaction mixture to assemble the gene.

30

24. The process of claim 1, wherein the oligonucleotides are annealed in blocks, said blocks are subsequently annealed and ligated together to form the intact gene.

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25. The process of claim 1, wherein the gelonin gene is constructed by a solid phase assembly process comprising the steps of:

a. attaching a synthetic oligonucleotide at or near one of its ends to a solid phase support material;

5

b. washing away excess, unbound oligonucleotide;

10

c. adding a molar excess of oligonucleotide, which possesses perfect base sequence complementarity with the support-bound oligonucleotide, throughout part or all of its length, and annealing the strands together to form a duplex DNA structure, then washing away excess, unbound oligonucleotide;

d. repeating step c until the entire gene has been assembled;

15

e. treating the assembled gene with DNA ligase to form an intact duplex DNA; and

f. releasing the synthetic gene from the support with a restriction enzyme or other suitable means.

20

26. The synthetic DNA of claim 1 containing a sequence of nucleotides coding for a protein gelonin or for a polypeptide which inhibits cellular protein synthesis but does not bind to a cell surface receptor.

25

27. The synthetic DNA of claim 26, wherein said nucleotide sequence is SEQ ID NO. 1 and fragments and derivatives thereof.

30

28. The synthetic DNA sequence of claim 27 wherein said DNA sequence encodes a protein which inhibits cellular protein synthesis but does not bind a cell surface receptor.

29. An expression vector containing a DNA of claims 26 or 27.

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30. The vector of claim 29, wherein said vector is pKK223-3.

5 31. The vector of claim 29, wherein said vector is pKC30 or one of its derivatives.

10 32. A host cell containing and expressing a recombinant DNA molecule of claims 26 or 27, wherein said host cell comprises a vector comprising the following elements linked sequentially at appropriate distances for allowing functional expression of DNA: a promoter; signal sequence elements; and a DNA sequence encoding gelonin.

15 33. The host of claim 32 wherein said host is selected from the group consisting of bacteria, fungi, insect cells, animal and plant hosts.

20 34. The host of claim 32, wherein said host is selected from the group consisting of strains of *E. coli*, *Pseudomonas*, *Bacillus* and yeast.

35. The host of claim 32 wherein said host is selected from the group consisting of mouse, swine, and human tissue cells.

25 36. The host of claim 34, wherein the *E. coli* is *E. coli* JM105.

30 37. The synthetic DNA of claim 27, wherein said DNA sequence is operatively linked to an expression control sequence which controls the expression of genes of prokaryotic or eukaryotic cells or their viruses.

38. The synthetic DNA of claim 37, wherein said expression control sequence is selected from the group consisting of an

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early promoter SV40, a late promoter SV40, a loc system, a TAC system, a TRC system, a TRP system, major operator and promoter regions of phage lambda and control regions of fd coat protein.

5 39. The synthetic DNA of claim 27, further comprising fusing said gene to a DNA sequence encoding a polypeptide domain that binds specifically to a cell surface receptor present on selected target cells.

10 40. A process for producing the synthetic DNA sequence of claim 26, comprising the steps of:

15 a. designing a double-stranded DNA fragment containing a sequence of three base codons corresponding to the primary amino acid sequence determined from purified gelonin protein so that said DNA sequence has features that facilitate synthesis, cloning, expression or biochemical manipulation of the gene;

 b. designing, synthesizing, purifying, the 5'-phosphorylating a set of synthetic oligonucleotides which are capable of being linked together to assemble the synthetic gene;

20 c. annealing and ligating the oligonucleotides together to assemble the synthetic gene.

 41. A process of producing the synthetic DNA sequence of SEQ ID NO. 1 and fragments and derivatives thereof of claim 27 comprising:

25 a. designing a double-stranded DNA fragment containing a sequence of three base codons corresponding to the primary amino acid sequence determined from purified gelonin protein so that said DNA sequence has features that facilitate synthesis, cloning, expression or biochemical manipulation of the gene;

30 b. designing, synthesizing, purifying, the 5'-phosphorylating a set of synthetic oligonucleotides which are capable of being linked together to assemble the synthetic gene;

c. annealing and ligating the oligonucleotides together to assemble the synthetic gene.

42. An immunotoxin comprising:

5

- (a) the product of the process of claim 41 and
- (b) an antibody conjugated to said product.

43. A process for producing, cloning, expressing and purifying a synthetic gene encoding gelonin, comprising the steps of:

10

a. designing a double-stranded DNA fragment containing a sequence of three base codons corresponding to the primary amino acid sequence determined from purified gelonin protein so that said DNA sequence has features that facilitate synthesis, cloning, expression or biochemical manipulation of the gene;

15

b. designing, synthesizing, purifying and 5'-phosphorylating a set of synthetic oligonucleotides which are capable of being linked together to assemble the synthetic gene;

c. annealing and ligating the oligonucleotides together to assemble the synthetic gene;

20

d. ligating the synthetic gene together with a suitable cloning vector;

e. determining the nucleotide sequence of the cloned gene to verify the correctness of the gene;

25

f. conducting site-directed mutagenesis to correct any undesired mutations in the cloned gene;

g. subcloning the gene into an expression vector;

30

h. introducing the expression vector bearing the synthetic gelonin gene into an expression host, wherein said host cell comprises a vector comprising the following elements linked sequentially at appropriate distances for allowing functional expression of DNA: a promoter; signal sequence elements; and a DNA sequence encoding gelonin;

j. isolating and purifying the gelonin from cells
5 expressing the gene.

(a) the product of the process of claim 43; and
(b) an antibody conjugated to said product.

(a) isolating vector DNA bearing the gene of claim 1;

20 46. A method for correcting mutations arising in the cloned gene of claim 1 by site-directed mutagenesis, further comprising the steps of:

(c) elongating the corrective oligonucleotide primer annealed to said mutation-bearing template strand by action of a DNA polymerase;

(e) isolating vector DNA; and

- (f) subcloning the corrected gene into an expression vector.

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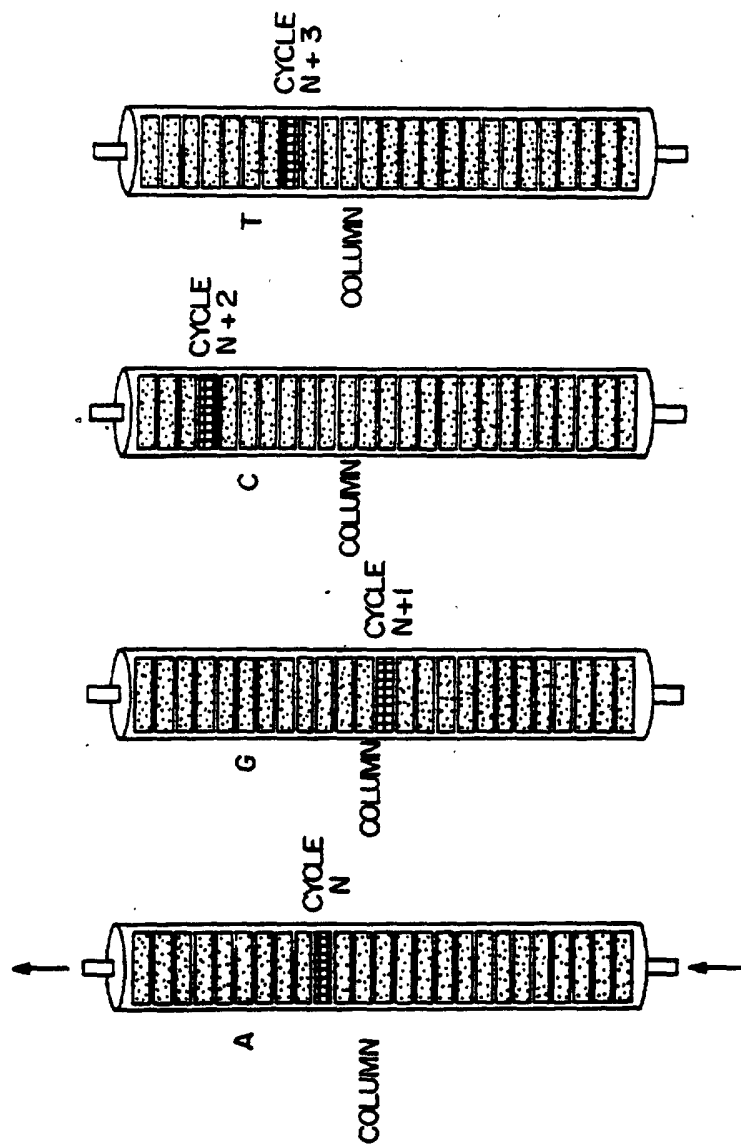
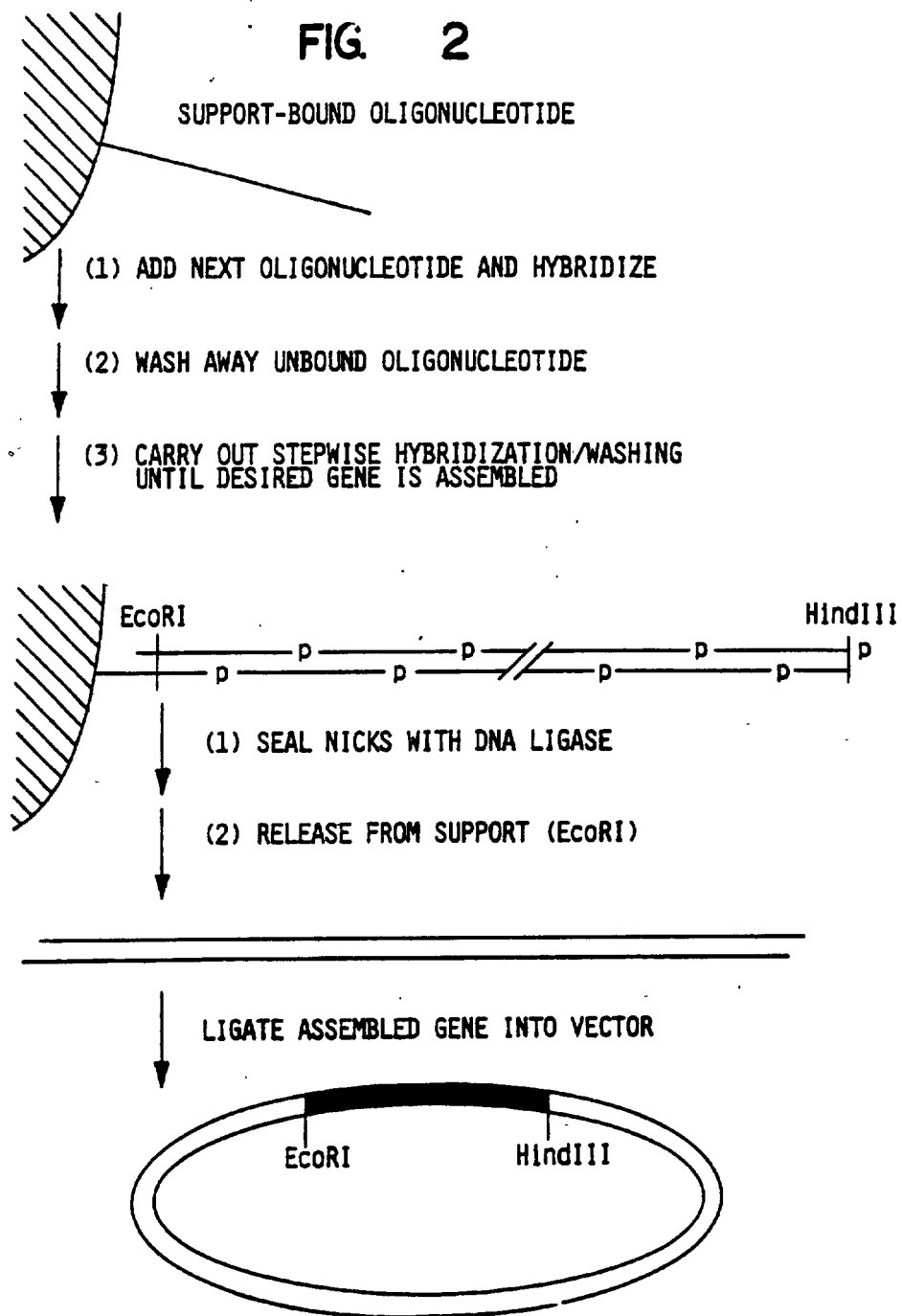


FIG. 1

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FIG. 2



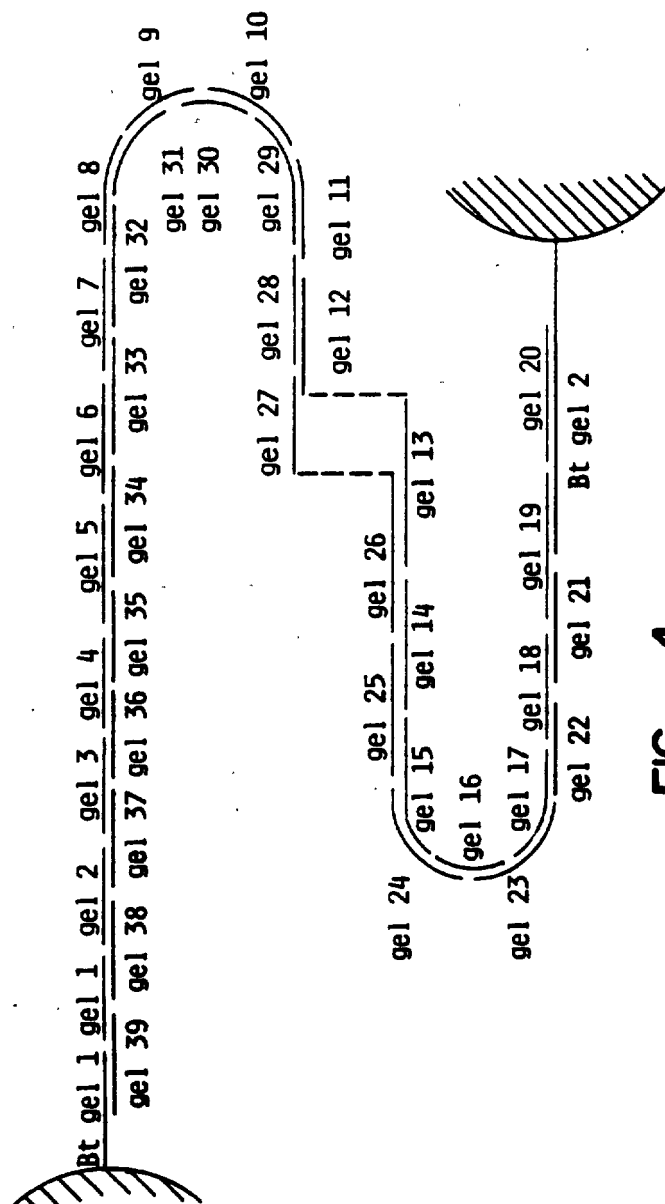


FIG. 4

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NAME OF GENE: GELONIN

oligo name length SEQUENCE (5'→3')

FIG. 5

1. Btge1 45+bt
 2. Btge12 61+bt
 3. ge11 40+p
 4. ge12 40+p
 5. ge13 40+p
 6. ge14 40+p
 7. ge15 40+p
 8. ge16 40+p
 9. ge17 40+p
 10. ge18 40+p
 11. ge19 40+p
 12. ge110 40+p
 13. ge111 40+p
 14. ge112 40+p
 15. ge113 40+p
 16. ge114 40+p
 17. ge115 40+p
 18. ge116 40+p
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 22. ge120 30+p
 23. ge121 40+p
 24. ge122 40+p
 25. ge123 40+p
 26. ge124 40+p
 27. ge125 40+p
 28. ge126 40+p
 29. ge127 40+p
 30. ge128 40+p
 31. ge129 40+p
 32. ge130 40+p
 33. ge131 40+p
 34. ge132 40+p
 35. ge133 40+p
 36. ge134 40+p
 37. ge135 40+p
 38. ge136 40+p
 39. ge137 40+p
 40. ge138 40+p
 41. ge139 54+p

Bt-TTTCGACGGTTCGACGGTTCGCGGATTCAGATATGGGTCGGATA
 Bt-TTTCGACGGTTCGACGGTTCGCGTTCAGAGCTTCACATATCCGGGCTTTGTCCACGAATTT
 D-CCGTAGCTTCAGCACCAAGGCGGACCTACATCACCTA
 D-CGTTAATCTTCGACGAATTCGCTTAACTGAACCG
 D-GAAGGTACAGCCATGGCATCCCGTTCGCTGAAGG
 D-ATGACCCGGGTAAATGCTTCGCTGGTGGGCTGAGCAA
 D-CGATAACGGTCAGCTGGCAGAAATCGCAATCGATGTACC
 D-AGCGTGTACGTAGTTCGCTATCAGGTTCGCTAACCGAGCT
 D-ACCTTCCTCAAGATGCTCCGGATCGAGCTACGAAGGCTT
 D-GTTCAAAACACCATCAAAACCCCTGCTGTTTGGTGGC
 D-AAATCTGCTTCGCTTCGGTGGCAGCTATCCGAGCTGG
 D-AAGGCGAAAGGCTACCGGAAATACCTGATCTGGGTAT
 D-CGAACCGCTTCGCTTCGGCATCAAAATCGCAATCGGACGAAAC
 D-GCGATCGACAACTACAAACCGACCGAATTCGCGAGCTCTC
 D-TGCTGGTTGTGATCCAGATGGTGGCAGAGCGGACGTTT
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 D-CCGAGTGCAGACGAGTTTTCGACGAGCTTCGATCGATC
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 D-CTGCGAGCTGACCGTTATCTGCTCAGCGCTACGAGCAG
 D-GAAGCATTTACCGGGTTCATACCTTACGAGCAGCGGG
 D-ATGCCATGGCTGTTACCTTCGCTTTCAGTTTAAACAGCA
 D-GTTCGTTCAAGAGTTAACGTTAGGTGATGAGGTTCGCGCC
 D-TTTGGTGTGAAGCTAACGGTATCCAGACCCATATGCTGAATTCGCGACGCGTGC

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/07066

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/ 69.1, 172.3, 240.1, 240.2, 243, 252.3, 252.33, 320.1; 530/ 370, 391.7; 536/27; 935/9, 10, 18, 23, 28, 29, 34, 44, 66

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Journal of Biological Chemistry, Volume 255, No. 14, issued 25 July 1980, P. Stirpe et al., "Celastrol, a New Inhibitor of Protein Synthesis, Nontoxic to Intact Cells, pages 6947-6953, entire document.	6, 10, 13, 15, 18, 20, 21-22 5, 7, 9, 11-12, 14, 16-17, 19, 21, 23-42, 44, 46-47
X Y	Nature, Volume 339, issued 01 June 1989, V. K. Chaudhary et al., "A recombinant immunotoxin consisting of two antibody variable domains fused to <i>Pseudomonas</i> exotoxin", pages 394-397, entire document.	26, 29, 32-33 30-31

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents	T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	A	document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

28 October 1992

Date of mailing of the international search report

18 NOV 1992

Name and mailing address of the ISA/
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

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Form PCT/ISA/210 (second sheet)(July 1992)*

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/07066

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Gene, volume 60, issued 1987, M.A. Wosnick et al., "Rapid construction of large synthetic genes: total chemical synthesis of two different versions of the bovine prochymosin gene", pages 115-127, entire document.	1-5, 7, 9, 11-12, 14, 16-17, 19, 21, 23-24, 26-39, 41-42, 44, 46-47
Y	Nucleic Acids Research, Volume 15, No. 12, issued 1987, Z. Homomský et al., "Solid-phase assembly of cow colostrum trypsin inhibitor gene", pages 4849-4856, entire document.	25
X	US, A, 4,888,415 (Lambert et al.) 19 December 1989, entire document.	8, 43, 45
Y	J. Sambrook et al., "MOLECULAR CLONING, A LABORATORY MANUAL" published 1989 by Cold Spring Harbor Press (N.Y.), pages xi-xxviii, entire document.	1-5, 7, 9, 11-12, 14, 16-17, 19, 21, 23-24, 26-42, 44, 46-47
Y	S. Berger et al. (eds.), "GUIDE TO MOLECULAR CLONING TECHNIQUES" (Methods in Enzymology, Volume 152), published 1987 by Academic Press, Inc (Calif.), pages v-x, entire document.	1-5, 7, 9, 11-12, 14, 16-17, 19, 21, 23-24, 26-42, 44, 46-47
Y	Nucleic Acids Research, Volume 16, issued 1988, S-I. Aota et al., "Codon usage tabulated from the Genbank Sequence Data", pages r315-r391, entire document.	11-12, 14, 16-17, 19, 21

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/07066

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (5):

C12P 21/02; A61K 39/44; C07H 21/00; C07K 13/00; C12N 1/00, 1/21, 5/10, 15/10, 15/63

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/ 69.1, 240.1, 240.2, 252.3, 252.33, 320.1; 530/ 370, 391.7; 536/27

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG files 155, 73, 5, 357 (Medline, Embase, Biosis, Biotech Abs.) Genbank, EMBL, Swissprot, PIR, search terms: gelonin, immunotoxin, oligonucleotide, ligation, gene(s), artificial, codon, optimiz?, synthetic, solid phase

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